Gene expression profiling in Osteosarcoma

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Submitted to the University of London for the degree of Doctor of Medicine

2006

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Abstract

Purpose: The aim of the work described in this thesis was to determine whether needle core and open biopsies from osteosarcoma (OS) provide sufficient quality of mRNA for cDNA array analyses which might then provide insights into the expression profile of OS.

Experimental Design: Sixteen samples collected from OS and two established cell lines were used for array analyses. A primary cell culture was also established from one of the OS biopsies. Total RNA was extracted and probes generated for cDNA arrays. A reference probe was included for computational analyses.

Results: cDNA probes were made for twenty five samples. Two of these samples were needle core bone biopsies. Twenty two cDNA probes were used for the generation of microarray data. Previous established statistical analysis confirmed the reliability of array data obtained in sixteen of the twenty two samples. Known genes involved in bone metabolism, osteoblast differentiation and cancer cell growth, were identified as up- or down-regulated in OS.

Conclusions: Without amplification of RNA, OS tissue including small core bone biopsies, are amenable to cDNA array analysis. Known and novel putative markers for OS, that could have prognostic value, were identified.

Acknowledgements

The work in this thesis was carried out whilst I was a clinical research fellow at University College London and the Royal National Orthopaedic Hospital.

Dr Ann Sandison was the driving force behind the need for molecular biological research within the London Bone and Soft Tissue Sarcoma group. She had meticulously collected and stored OS samples which allowed me to start my research on this rare primary bone tumour. Sadly she left the department for a new post and I missed her enthusiasm and support for the work but will always remain indebted to her for making it a reality.

With thanks to Prof Chris Boshoff and Dr Jeremy Whelan for their supervision and extreme patience.

To Mike Kaiser at Institute of Orthopaedics and Musculo-Skeletal Science, University College London (UCL) and the Royal National Orthopaedic Hospital NHS Trust, Brockley Hill, Stanmore for all the work relating to characterisation and staining of primary cultured cells.

Prof Mike Horton and Dr Jo Price for their interest and support in understanding normal bone physiology and giving me vital contacts to pursue to help me complete my laboratory work.

Dr Brendan Jackson, University of Sheffield for performing the osteocalcin analysis.

Dr Lucy di Silvo, Institute of Orthopaedics and Musculo-Skeletal Science University College London and the Royal National Orthopaedic Hospital NHS Trust, Brockley Hill, Stanmore for donation of primary human osteoblasts.

Caroline Clifford for her time and teaching particularly with alkaline phosphates staining.

Prof Allen Goodship for his support in completing this work.

Ms Natalie Wilder for Actin primers, WIBR UCL.

Dr Stoyan Radkov, WIBR UCL for the Saos 2 cells used.

Dr John Bennet, Royal Eastman Dental Institute, UCL for the donation of MG63 cells.

Dr Richard Lamb, ICR for the use of his ezrin antibody.

The orthopaedic surgeons Yohan Witt, Steve Cannon, Tim Briggs and especially Justin Cobb who were so enthusiastic and obliging with their theatre time in alerting me to new cases and then allowing me collect fresh, correctly stored precious OS samples.

With thanks to Dr Stephen Henderson for all his efforts in managing not only the huge spreadsheets of gene expression data generated but coping with my rudimentary understanding of statistics.

I am indebted to my laboratory ally, Dr Tyson Sharp who unselfishly gave his time and expertise in teaching me all the bench work and tissue culture required to get me started. He also shared his computer with me and taught me all the software packages to collate, impute and interpret my data.

And finally my long suffering and endlessly supportive husband Con, without whom I am nothing.

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Leonard P, Sharp T, Henderson S, Hewitt D, Pringle J, Sandison A, Goodship A, Whelan J, Boshoff C.

Gene expression array profile of human osteosarcoma (attached copy at back of thesis)

Br J Cancer. 2003 Dec 15; 89(12):2284-8.

Wolfson Institute for Biomedical Research, University College London, Gower Street, London, UK.

Radkov S, Sharp T, **Leonard P**, Boshoff C. (Abstract) Gene expression profiling of REF cells transfected with LNA. Proceedings of 3rd International KSHV meeting. Boston USA 2000

List of Abbreviations

aH	atypical endometrial hyperplasia
ABL	Abelson murine leukaemia
Ag	Antigen
ALT	Alternative lengthening of telomeres
ATM	Ataxia-telangiectasia
ATP	Adenosine 5'-triphosphate
APE	Apurinic endonuclease
B-CLL	B cell chronic lymphatic leukaemia
bFGF	basic fibroblast growth factor
BKV	T antigen of BK virus
β-ME	β-mercaptoethanol
BMP	Bone Morphogenetic Proteins
BMU	Basic multicellular units
Bp	Base pair
CAK	cyclin activated kinase
CAM	Cell adhesion molecule
Cbfa1	Core binding factor alpha1
CBP	CRE-binding protein
CDC	Cell division cycle
Cdk	cyclin-dependent kinase
cdkI	cdk inhibitor
cDNA	Complimentary deoxyribose Nucleic acid
cH	Complex endometrial hyperplasia
dATP	Deoxyadenosine triphosphate

dCTP	Deoxycytidine triphosphate
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dTTP	Deoxythymidine triphosphate
ECF	Extracellular fluid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EFS	Event free survival
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EM	Electron Microscope
ERCC	Excision repair cross-complementing
ERM	ezrin, radixin, and moesin
ER	Oestrogen receptor
ERBB2	Epidermal growth factor type 2
ESFT	Ewing's sarcoma family of tumours
EST	Expressed sequence tag
FADD	Fas-associated death domain
FasL	Fas ligand
FCS	Fetal calf serum
FGF	Fibroblast Growth Factor
FLICE	Cellular Fas-associated death domain-like interleukin-1-beta converting enzyme
FLIP	Flip inhibitor protein

GADD	Growth arrest and DNA inducible gene
GC	Guanine-cytosine
GEM	Gene expression microarray
GM-CFU	Granulocyte-macrophage colony forming unit
H&E	Haematoxylin and Eosin
HCL	Hydrochloric acid
HER-2/neu	erbB-2 gene
IAP	Inhibitor of apoptosis protein
IFP	Human interferon-induced leucine zipper protein
IRF-4	Interferon regulatory factor-4
IMS	Industrial methylated spirits
IL-12	Interleukin -12
IGF	Insulin-like growth Factors
IGFBP3	Insulin-Like Growth Factor Binding Protein 3
JCV	T antigen of JC virus
LCM	Laser capture microdissection
LLNL	Lawrence Livermore National Laboratory
LOH	Loss of heterozygosity
M	Mitosis
MAP	Mitogen Activated Promotor
MDR	Multidrug resistance
MDT	Multidisciplianry Team
MM	Mismatch
MMP	Matrix metalloproteinases
MPF	Maturation-promoting factor
MPM2	M-Phase phosphoproteins 2

mRNA	Messenger RNA
MUM1	multiple myeloma oncogene 1
MTP-PE	Muramyl tripeptide phophatidylethanolamine
N-CAM	Neural cell adhesion molecule
NER	Nucleotide excision repair
OB	Osteoblast
OCL	Osteoclast
OS	Osteosarcoma
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PIP_2	Phosphatidyinositol 4-5 biphosphate
POG	Paediatric Oncology Group
PLM	Phospholemman
PM	Perfect match
PMT	Photomultiplier tube
PKC	Protein kinase c
PDGF	Platelet Derived Growth Factor
pRB	Retinoblastoma protein
PTH	Parathormone
RANK	Receptor activator of NF-kappaB
RANKL	Receptor activator of NF-kappaB ligand
RMS	Rhabdomyosarcoma
RNA	Ribonucleic acid
RNOH	Royal National Orthopaedic Hospital

RTK	Receptor tyrosine kinases
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RT	Reverse transcription
S	Synthetic
SAGE	Serial analysis of gene expression
SDS	Dodecyl Sulphate
sH	simple endometrial hyperplasia
SOM	Self organising map
STAT	Signal transducer and activator of transcription
SV40	Simian virus 40
TAE	Tris-EDTA
TDG	Thymine DNA glycosylase
TGF β	Transforming Growth Factor β
TK	Tyrosine Kinases
TNF	Tumour necrosis factor
tRNA	Transfer RNA
TS	Thymidylate synthase
TSG	Tumour suppressor genes
UEC	Uterine endometrial carcinoma
UCL	University College London
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

Chapter 1 Background

Introduction

Cancer is a multi-faceted disease that presents many challenges to clinicians and research scientists looking for a better understanding of the disease process and through this knowledge hopefully better ways to combat its devastating effects. One of the central challenges for researchers working in this field is to identify specific markers for each type of cancer that can improve diagnosis and assist in the development of more effective targeted treatments. The great majority of cancers develop sporadically so the understanding of some of the somatic mutations that arise from single normal cells which then afford those cells an advantage in terms of growth and survival can help scientists put together proposed pathways of tumourigenesis. The best known work regarding the accumulation of somatic mutations was by Bert Vogelstein, who described the series of steps which transformed normal benign colonic epithelium to invasive colon cancer [1, 2].

A number of cancer-related genes have been detected, but at present tumour classification is still largely based on histology. Despite two patients having apparently the same diagnosis their clinical course can run very differently. To date we rely on prognostic factors such as oestrogen receptor negativity in breast cancer or mucoid differentiation in colon cancer to determine poor outcome. Some predictive factors have been discovered such as HER2/NEU receptor positivity and oestrogen receptor positivity (ER) in breast cancer [3] and thymidylate synthase expression (TS) in colon cancer [4], which can help clinicians choose the most effective treatment for their patients. Despite all the advances in treating cancer patients, additional methods for classifying cancers are urgently needed. This approach if successful will allow the clinician not only to make an accurate diagnosis by determining the signature of that

cancer but also exploit both the tumour and patient factors to allow individualised treatments.

The massive amount of deoxyribose nucleic acid (DNA) sequence material available as determined by the Human Genome project [5, 6] and the development of technologies to exploit this knowledge have coined a new term "Genomics". This term applies to any high throughput technology such as microarray analysis or large scale sequencing to detect single nucleotide polymorphisms (SNPs). If used in the context of the analysis of gene expression data it is called "transcriptomics". The transcription of genomic DNA to produce mRNA is the first step in the process of protein synthesis [7]. Measurement of gene expression can reveal important information about not only the phenotypic and morphological properties of the tissue being analysed but the cellular responses to external stimuli.

There is a great deal of interest in the use of functional genomics to identify cancer associated genes and their protein products. This information will have the potential to identify markers for early detection, classification and prognosis of cancers, as well as pinpointing agents for improved treatment outcomes.

This thesis will look at a very rare but serious cancer, osteosarcoma (OS). It is a primary bone tumour which has a distinct peak of incidence in adolescence. Modern combination regimens of cytotoxic treatment have improved the overall outlook for those affected but over 40% will still succumb to their disease despite intensive treatment. OS has many different sub-types which are not always easy to differentiate by routine histological methods. Some variants run a much more indolent course,

whilst others are highly aggressive. There is an urgent need for better identification of subtypes and prognostic and predictive information as soon as the diagnosis is made to spare unnecessary toxic treatments and to optimise current therapies.

This work was undertaken as part of the UK regional Bone and Soft Tissue Sarcoma unit combining the clinical expertise of oncologists, radiologists, pathologists and sarcoma surgeons to provide accurate patient histories and fresh OS samples. The scientific expertise at the Wolfson Institute of Biomedical Research (WIBR) enabled this translational research. The thesis will outline the problems presented by those individuals affected by OS and the current management strategies. It will discuss the current knowledge base of aetiological, prognostic and predictive factors. It will discuss the application of nylon cDNA microarrays which contain over 5000 known genes to a cohort of patients from this unit and discuss the information learnt from this work.

1.1 Cancer cell Biology

The hallmarks of a malignant cancer cell are uncontrolled cell proliferation, invasiveness and the ability to spread or metastasise to distant parts of the body. The development of cancer in humans involves a complex succession of events that usually occur over many decades. During this multistep process the normal genetic make up of the incipient cancer cell acquires abnormal or mutant alleles of genes that are either positive effectors of transformation or negative growth regulators. The gain of function mutations can activate proto-oncogenes leading to cell proliferation [8]. The loss of function of tumour suppressor genes (TSG) whose products are negative growth regulators, lead to carcinogenesis as the normal cell checkpoints are lost. Cancer invasion can be seen as a derangement in the proper sorting of cell populations, causing a violation of normal tissue boundaries. Tissue architecture is normally maintained by the basement membrane. It delineates the tissue boundaries and facilitates the cell to cell communication with the surrounding extracellular matrix (ECM) [9].

The ability of the cell to produce exact copies of itself during replication is an essential component of cellular life [7]. The molecular machinery of cell cycle control is highly organised and has been relatively conserved through evolution [10]. Intracellular and extracellular factors influence this process as do genetic factors.

How do cells become malignant? Cancer cells have the ability to generate their own mitotic signals, to resist exogenous growth-inhibitory signals, to evade apoptosis, to proliferate uncontrollably, to acquire vasculature and to invade and metastasise [9, 11, 12].

1.1.1 Control of the cell cycle

The normal mechanism which allows a cell to replicate and then divide requires two functional and two preparatory phases. This first phase, also known as the S (synthetic) phase, is where DNA replicates itself. This is followed by the M phase or mitotic phase where the accurate segregation of duplicated sets of chromosomes occurs. Before the S phase the cell prepares itself biochemically in a resting phase referred to a G_1(gap 1) and before the M phase another poorly understood resting phase known as G_2 (gap 2). Cells that are not actively dividing may be permanently removed from this cycling phase by terminal differentiation or be temporarily arrested by moving into a non-cycling state referred to as G_0. These events normally occur in an orderly fashion where division does not proceed until replication is complete. External stimuli such as growth factors, cell to cell contact, nutrients and mitogens influence this process. The complete cell cycle is represented schematically in Figure 1.[10].

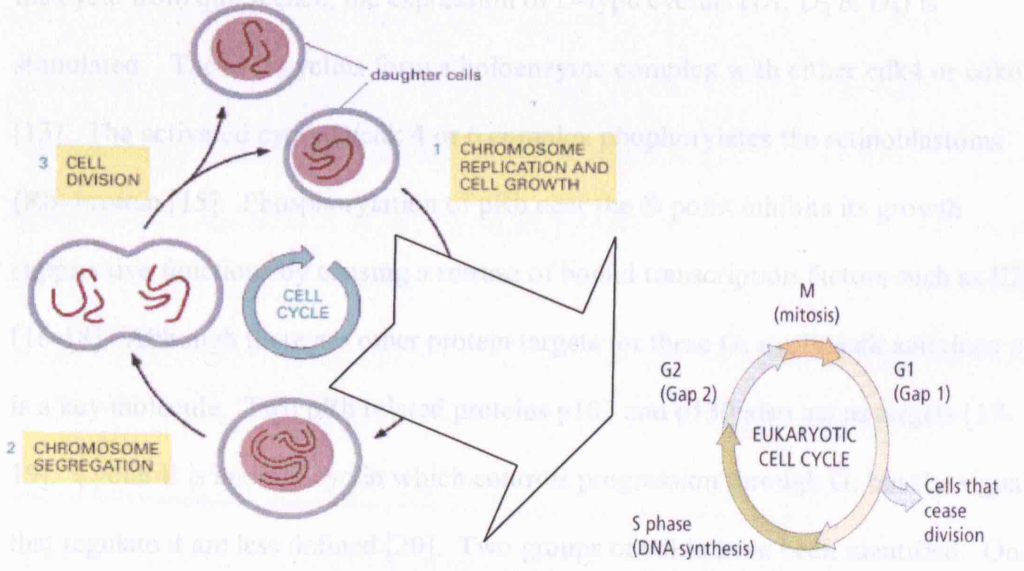


Figure 1 - Schematic representation of cell division

Key transitions in the cell cycle occur when the enzymatic activity of specific intracellular enzymes known as cyclin-dependent kinases (cdks) activates the proteins required for progression from one phase to the next. Cyclins D and E are synthesised in G_1, cyclin A in S and G_2 and cyclin B in G_2 and M. Cyclins form complexes with a cdk and become activated by regulated kinase activity (CAK). Activated cdk phosphorylates target gene products required for the next phase of the cycle. Phosphorylation of the ATP binding site, or reactivation by phosphatases, leads to deactivation of the cdks. Cyclin-dependent kinase inhibitors (cdkI) can either block assembly of the cyclin/cdk complex or prevent activation of the cdk kinase [13].

Molecular events in G_1 prepare the cell for DNA synthesis. There is a point in G_1 known as the restriction point ®, after which the cell is committed to progress to the S phase. After this point mitogenic factors are no longer required for the cells to complete DNA synthesis [7, 14]. When mitogenic signals stimulate the cell to enter the cycle from quiescence, the expression of D-type cyclins (D_1, D_2 & D_3) is stimulated. These D-cyclins form a holoenzyme complex with either cdk4 or cdk6 [13]. The activated cyclinD/cdk 4 or 6 complex phophorylates the retinoblastoma (Rb) protein [15]. Phosphorylation of pRb near the ® point inhibits its growth suppressive functions by causing a release of bound transcription factors such as E2F [16-18]. Although there are other protein targets for these G_1 cyclin/cdk activities pRb is a key molecule. Two pRb related proteins p107 and p130 also act as targets [17-19]. Cyclin E is another cyclin which controls progression through G_1 but the signals that regulate it are less defined [20]. Two groups of cdkIs have been identified. One contains the inhibitors p21 $^{WAF1/CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$. These inhibit the kinase activities of cyclin D/cdk4, cyclin D/cdk6, cyclin E/cdk2 and cyclinA/cdk2 as well as preventing cdk activation by CAK [21]. These three inhibitors therefore prevent

progression from G1 to S. p53 increases expression of p21 $^{WAF1/CIP1}$ whereas p27^{KIP1} is induced by TGFβ. The second cdkI family contains the inhibitors p16^{Ink4}, p15^{Ink4B}, p18^{Ink4C} and p19^{Ink4D} which also mediate through TGF β [22].

The S phase heralds the onset of DNA replication. Briefly DNA replication starts with a partial unwinding of the double helix at an area known as the replication fork. This unwinding is accomplished by an enzyme known as DNA helicase.

As the two DNA strands separate and the bases are exposed, the enzyme DNA polymerase moves into position at the point where synthesis will begin. The sum of all genes in a human cell in the human genome is estimated to be approximately 3 billion base pairs [23]. There are three DNA polymerases (ά, δ and ε) [7]. Although these enzymes copy with high accuracy, they usually incorporate one incorrect nucleotide per 10^5 or 10^6 copies. Misincorporated nucleotides are removed by proofreading 3' to 5' exonuclease activities. There is a final repair mechanism which removes mismatched DNA bases. These processes together reduce the potential mutations by 1000 fold. DNA is a double helix, thus both strands are copied simultaneously. One strand acts as the leading strand as its DNA is copied continuously. The other is termed the lagging strand as it is copied discontinuously. The process therefore ends with DNA elongation to ensure all replicons are the same length. Telomeres are nucleoprotein structures that cap chromosome ends, protecting cells from inducing inappropriate DNA damage responses and from chromosomal loss and recombination. With each round of replication, telomeres erode because of the inability of DNA polymerases to replicate the very ends of DNA. When telomeres become critically short, senescence or apoptosis is induced [7, 24]. Tumours are able to produce the enzyme telomerase to maintain telomeres as so escape senescence and

become immortal [25-27]. This is a critical step in cancer development and is discussed later in Section 1.3.2.3.

This replication phase is followed by gap 2 where the two copies of the genomes are segregated into daughter cells. A complex of cyclin B/cdk2 is the major regulator of this transition. The complex is known as maturation-promoting factor (MPF) [28]. The targets for this complex are not known for entry in M [29] but both MAP kinase and MPM2 kinase appear to be phophorylated by this complex [30].

1.1.2 Extracellular signals

Many extracellular factors such as nutritional status, cell-cell contact and extracellular peptides influence intracellular events. Examples of external factors include growth factors which drive cells in the resting phase G_0 to proceed through the cell cycle. Cytokines are soluble mediators of cell-cell communication and include interleukins, interferons and colony stimulating factors. In general, cytokines bind to specific cell surface receptors and initiate a cascade of biochemical events that influence intracellular events usually ending in the repression or transcription of various genes.

Protein tyrosine kinases (TKs) are enzymes that catalyse the transfer of phosphate from ATP to tyrosine residues in polypeptides. The human genome contains about 90 TK genes and 43 TK –like genes, the products of which regulate cellular proliferation, survival, differentiation, function and motility [31]. TKs are now considered excellent targets for cancer chemotherapy. Imatinib is the most successful of the commercially available TKIs as in addition to its ability to inhibit the abl kinase it also inhibits the PDGF-R and C-KIT tyrosine kinases [32, 33].

1.1.3 Cell cycle checkpoints

These are mechanisms controlled by certain gene products which determine if normal cell cycling proceeds. If any stresses such as DNA damage are detected, then inhibition of cell cycle progression occurs as these cell cycle checkpoints are invoked. The tumour suppressor gene p53 is a critical component of the pathway that results in G_1 arrest. DNA damage leads to an increase in the level of p53 which in turn activates transcription of the p21 $^{WAF1/CIP1}$ gene. Increased expression of p21 $^{WAF1/CIP1}$ results in an inhibition of cyclinE/cdk2 activity which prevents phosphorylation of pRb and therefore prevents progression from G_1 to S phase. In some instances the induction of p53 leads to programmed cell death or apoptosis. A high proportion of cancers carry a mutated p53 which leads to the loss of the normal function of p53 [34, 35]. This in turn leads to the inappropriate replication of damaged DNA, or inappropriate survival of cells that normally would undergo apoptotic death. Other checkpoints are less well understood but it is thought that the mutated ataxia-telangiectasia (ATM) gene is one of the controlling checkpoints in G2 [36, 37]. This checkpoint is of interest to radiobiologists as it is thought there is a link between G2 and radiosensitivity [38].

1.1.4 Apoptosis

Apoptosis is an energy dependent mode of cellular death that occurs in response to specific stimuli e.g. cytotoxic chemotherapy, ionising radiation or viral infection. It is also a critical event in normal development and normal tissue homeostasis. As has been described, gene products such as *p53* which control cell cycle checkpoints, influence apoptotic tendencies. In tumourigenesis it appears the loss of apoptotic signals may be the critical mutational event. *BCLl-2* is such an anti-apoptotic gene. The molecular characterisation of the t (14:18) chromosomal translocation commonly seen in follicular lymphoma led to its identification [39]. *BCL-2* is one of a family of proteins which interact with each other to influence apoptotic tendencies. When *BCL-*

2 is expressed at high levels it forms a complex with BAX, preventing BAX homodimerisation and inhibiting cell death. This over-expression of *BCL-2* in lymphomas leads to uncontrolled tumour growth. Many other anti-apoptotic proteins, including FLIP (Flice inhibitor protein) and IAP (inhibitor of apoptosis protein) are activated in common epithelial malignancies [40, 41].

1.1.5 Angiogenesis

When cancers proliferate they cannot grow to more than a few millimetres without acquiring access to a blood supply to sustain growth [42] because passive diffusion of nutrients and waste products is insufficient for a tumour's metabolic needs. Efficient transfer of metabolites requires the tumour cells to be within 200μm of a capillary vessel and this requires new vessels to expand towards the tumour foci. This acquisition of a blood supply is known as angiogenesis. Malignant tumours release angiogenic signals, which attract and stimulate endothelial cells. The endothelial cells then construct capillaries within the tumour mass and forge direct connections within the existing vasculature of the host, so providing vital nutrients and oxygen to the cancer cells and taking away the generated metabolic waste. These newly formed blood vessels are leakier than normal vessels and provide relatively easy access to the circulation for immediately adjacent tumour cells. The first described pro-angiogenic factors were vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in the early 1980's. Since that time 15 angiogenic factors have been identified and include Platelet-derived Growth Factor (PDGF), Hepatocyte growth factor and angiogenin amongst others. Thrombospondin-1 is one anti-angiogenic factor. In simplistic terms solid tumours achieve angiogenesis by secreting pro-angiogenic factors and down-regulating anti-angiogenic factors [43, 44]. The ability of a tumour to spread or metastasise is directly related to the number of microvessels growing in a tumour mass [45-47].

1.1.6 Metastasis

The growth of new leaky blood vessels into an enlarging cancer mass facilitates the dissemination of cancer cells into the circulation [48]. However they must be able to escape the cohesive constraints of the cancer mass before they can successfully metastasise [49, 50]. Cell to cell cohesion is maintained by active homotypic (like-binds-like) cell adhesion molecules (CAMs). The most important CAMs belong to the cadherin [51, 52] family of cell surface receptors. These cadherins are transmembrane glycoproteins with a calcium-dependent ability to mediate cellular aggregation. Down-regulation of cadherins results in loss of tissue integrity and is responsible for the breakdown of architecture which then leads to escape of cells into the circulation [52]. The activity of epithelial cadherin (E-Cadherin) in carcinomas may come from the mutation of the E-cadherin gene [53]. It is thought that cytokines released by the tumour probably during angiogenesis lead to the down-regulation of tissue specific cadherins, thereby modulating the cell to cell interaction and promoting the development of metastases [54].

Integrins are a family of adhesive glycoproteins which primarily recognise constituents of the ECM and are also involved in the migration of invading cancer cells through the surrounding tissue [55, 56]. The integrins are transmembrane heterodimeric glycoproteins formed from non-covalently associated α and β chains. The nature of the α and β constituents is responsible for the ligand specificity of these molecules and so determines to which of the substrates it binds [57]. For e.g. $\alpha_5\beta_1$ is the classical fibronectin receptor and cells expressing this heterodimer can attach to fibronectin [58, 59]. Integrins however have both adhesive and signalling roles during tumour angiogenesis [60]. Tumour cells up-regulate or maintain the expression of integrins that co-operate with receptor tyrosine kinases (RTKs) to promote tumour progression [61]. Integrins also mediate the formation of microemboli, which are

composed of tumour cells, platelets and leucocytes. They facilitate the adhesion of tumour cells to the endothelium which therefore promotes their docking and extravasation at the metastatic site.

Enzymes such as matrix metalloproteinases (MMPs) are proteolytic enzymes that can degrade all constituents of the ECM and so provide another means of cells migrating into the circulation. Tumours can produce MMPs to aid metastasis [62-65].

This is a concise overview of normal cellular processes which can be manipulated by tumour cells to proliferate, invade and metastasise. It is outside the scope of this thesis to give an exhaustive account of all known oncogenes, tumour-suppressor genes and signal transduction pathways described thus far for malignant solid tumours. This serves as a framework to understand some of the known and hypothesised processes which may be of important in the pathogenesis of OS.

1.2 Normal Bone Physiology

The skeleton is a dynamic specialised organ responsible not only for the shape, scaffolding and protection of the body, but comprises both mineralised and non-mineralised bone [66]. Cortical bone is the hard outer wall of normal bone, accounting for about 80% of its total mass with trabecular or cancellous bone forming the remainder. This inner spongy bone is often described as a three dimensional honeycomb network of trabeculae. It lies mainly within the metaphysis of long bones and is in intimate contact with the bone marrow.

Bone is made up of minerals (65%) calcium, magnesium, phosphorus and other ions required for normal homeostatic mechanisms, organic matrix (35%) and specialised cells such as osteoblasts, osteocytes and osteoclasts, and water. Hydroxyapatite is the main crystal structure woven between the collagen fibres. Collagen comprises almost 90% of the organic matrix with non-collagenous proteins osteopontin, osteonectin, osteocalcin, bone Gla protein and bone sialoprotein making up the rest. Some of these proteins may function in initiating mineralisation and binding of the mineral phase to the matrix [66].

There are three distinct embryological lineages that generate the skeleton [67]. The axial skeleton is generated by the somites, the lateral plane generates the limb skeleton and the branchial arch and craniofacial bones and cartilage arise from the neural crest.

The axial skeleton forms the central axis of the skeleton and comprises the skull, vertebral column and sacrum. The bones of the extremities including the scapula and pelvis are collectively referred to as the appendicular skeleton. The bones of the hands and feet are called the acral skeleton.

On the basis of their gross appearance, bones are divided into two main groups: flat and tubular bones. The bones of the trunk and craniofacial region generally tend to be classified as flat bones. The extremity bones and ribs tend to be tubular. The tubular bones are further sub-divided into the long bones (femur, tibia and humerus) and short bones (phalanges, metatarsals and metacarpals). The tubular bones have several regions or zones. The epiphysis is the region between the growth plate and the end of the bone in skeletally immature individuals or between the growth plate scar and the end of the bone in skeletally mature individuals. The metaphysis is the region adjacent to the growth plate opposite to the epiphysis. The shaft or diaphysis is the region connecting the metaphysis and the physis is the region of bone corresponding to the

growth plate. Figure 2 demonstrates the position of the two growth plates. Certain tumours have a predilection for particular regions of bone [67].

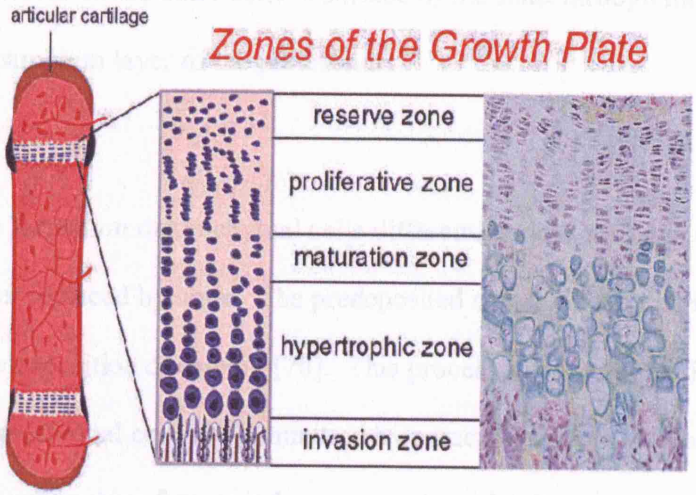


Figure 2 - Schematic representation of the growth plate in a long bone

There are two major modes of bone formation or osteogenesis, and both involve the transformation of a pre-existing mesenchymal tissue into bone tissue. These two processes are intramembranous ossification or enchondral bone formation. In the former, clusters of foetal mesenchymal cells differentiate directly into osteoblasts. They first appear as many separate centres of ossification that enlarge to fuse and form a single plate. This process occurs primarily in the bones of the skull [67]. The mechanism of intramembranous ossification involves the bone morphogenetic proteins (BMP) 2, 4 and 7 and a transcription factor called Core binding factor alpha1 (*CBFA1*) [67, 68]. *CBFA1* is now referred to as *RUNX2*. The BMPs activate *CBFA1(RUNX2)* which in turn activates genes for the extracellular matrix including osteocalcin, osteopontin. In the knockout mouse for *CBFA1(RUNX2),* the mice homozygous for this deletion died shortly after birth and were found to have a skeleton that completely lacked bone. Mice that were heterozygous for *CBFA1(RUNX2)* showed signs similar to the human condition cleidocranial dysplasia where the skull

sutures fail to fuse and individuals are born with either absent or deformed collar bones [69]. Growth in the diameter of bones formed this way continues principally by the deposition of osteoid on the outer convex surface of the shaft through membranous ossification in the cambium layer of the periosteum.

In enchondral bone formation mesenchymal cells differentiate into cartilage and this cartilage is then later replaced by bone. The predeposited cartilaginous matrix serves as a scaffold for the deposition of osteoid [70]. This process can be divided into five stages: At first mesenchymal cells are committed by paracrine factors to become cartilage cells. These paracrine factors induce mesenchymal cells to produce the transcription factors PAX 1 and scleraxis. In turn, these transcription factors are thought to activate cartilage specific genes [71] [72]. During the second stage, the committed mesenchymal cells condense into compact nodules and differentiate into chondrocytes. The initiation of these condensations is thought to be due to N-cadherin and neural cell adhesion molecule (N-CAM) seems to be essential in maintaining them [73]. The gene SOX9 in humans is expressed in the pre-cartilaginous condensations. Mutations of the human SRY-related gene, SOX9, located on chromosome 17, gives rise to a rare disorder called campomelic dysplasia, which gives rise to multiple skeletal deformities and the affected babies die from respiratory failure as their tracheal and rib cartilage are poorly formed [74]. The third and fourth stage comprises the chondrocytes proliferating and then enlarging in size. The large chondrocytes alter the matrix by producing collagen X and more fibronectin. This process allows mineralisation. Finally the cartilage is invaded by blood vessels and the chondrocytes die by apoptosis. As the cartilage cells die, the cells that have surrounded the cartilage differentiate into osteoblasts. This whole process of the predeposited matrix undergoing focal calcification followed by vascular invasion and resulting in bone

occurs at the mesenchymal-vascular junction of the epiphyseal growth plate and metaphyseal bone. Invasion of continually growing cartilage is rapidly followed by osteoblastic differentiation and deposition of osteoid. The devitalised, calcified cartilage serves as scaffolding for the deposition of bone matrix and is resorbed by osteoclasts at the same rate at which the growth plate is internally expanded. This means long bone growth occurs while the thickness of the epiphyseal plate remains constant. When the interstitial expansion of the epiphysis ceases, this leads to the termination of growth. Bones of the vertebral column, pelvis and limbs are formed in this way. If all our cartilage were turned into bones before birth we would not grow any larger.

The skeleton constantly undergoes remodelling, the process of replacement and renewal of bone. Osteoblasts (OB) are the cells that synthesise and secrete bone matrix which is then mineralised and osteoclasts (OCL) resorb bone. Tubulation and remodelling are achieved by OCL activity resorption on the inner concave surface.

OBs derive from mesenchymal stem cells (MSCs) located in the bone marrow [67]. MSCs contribute to the regeneration of mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, adipose and stroma [75]. In vitro differentiation of MPCs to OBs, chondroblasts, myoblasts or adipoblasts appears to be specific, as most cells recovered after 14-21 days of differentiation express lineage specific markers [76]. Figure 3 shows OBs in relation to the other cell types that differentiate from MSC. *CBFA1(RUNX2)* is one of the most important transcription factors required for OB differentiation [77, 78]. Other transcription factors include SMADs 1, 3 and 5 [79-81], menin [82] and Twists [83]. *CBFA1(Runx2)* target genes include genes expressed by mature OBs, such as osteocalcin, bone sialoprotein, osteopontin and collagen ά 1 [84]. The term OB refers to the whole spectrum of OB cells regardless of their stage of

differentiation. In their early stages they are highly proliferative non-bone forming cells. As they mature they become more cuboidal and are able to produce bone. Once they have completed their function of secreting bone they turn into elongated, flat mononuclear cells that become buried within the lacunae of the mineralised matrix. Once buried in the matrix they are termed osteocytes. There are ten times as many osteocytes as OBs. Growth factors such as *TGF-β.IGF-1, PDGF* and *FGF* are able to influence the replication and differentiation of committed OB progenitors toward the OB lineage [85].

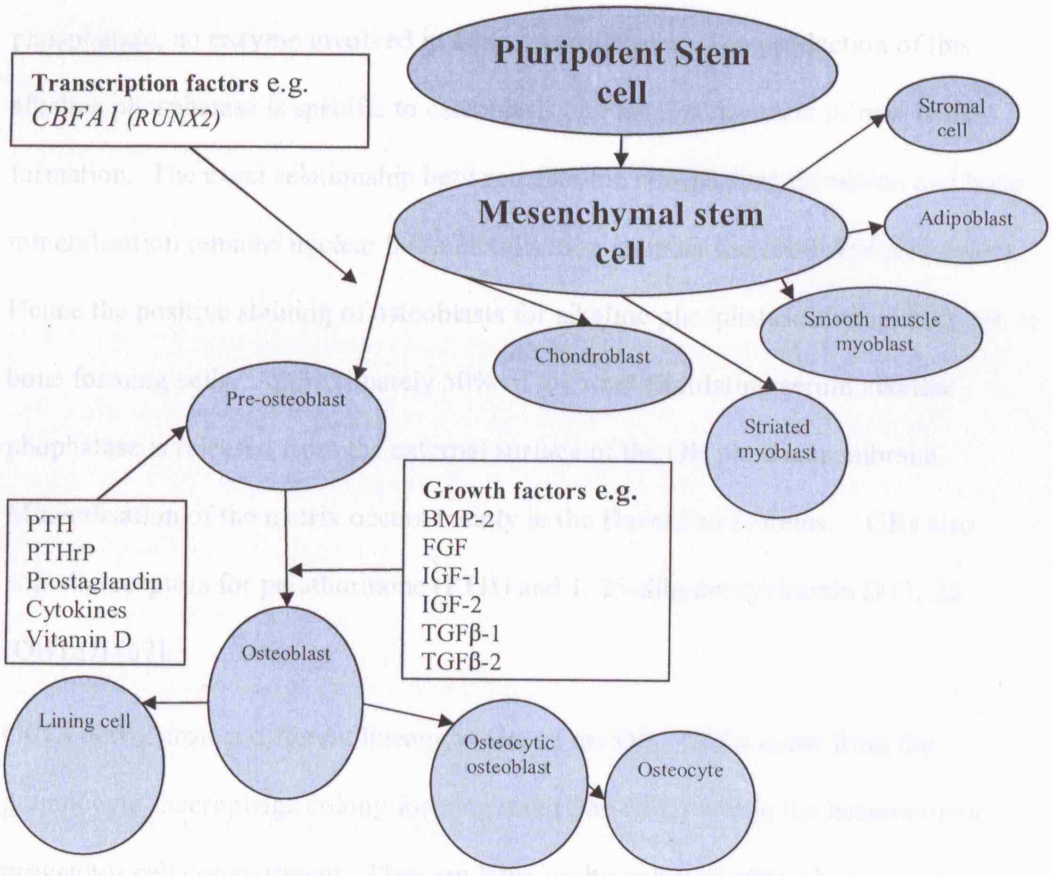


Figure 3 - Schematic representation of the growth plate in a long bone

The major product of bone forming OBs is type 1 collagen. They also synthesise a number of other proteins which are incorporated into the bone matrix, including osteonectin and osteocalcin, which constitute 40-50% of the non-collagenous proteins of bone [86]. The precise function of these proteins remains unknown. Other OS derived proteins include glycosaminoglycans, which are attached to one or two small core proteins: PG-1 (or biglycan) and decorin. Decorin has been implicated in the regulation of collagen fibrillogenesis. A number of other minor proteins such as osteopontin, bone sialoprotein, fibronectin, vitronectin and thrombospondin serve as attachment factors that interact with integrins.

OBs produce moderately high amounts of a specific skeletal form of alkaline phosphatase, an enzyme involved in bone mineralisation. The production of this alkaline phosphatase is specific to osteoblasts and heralds the onset of new bone formation. The exact relationship between alkaline phosphatase formation and bone mineralisation remains unclear but mineralisation is under the control of osteoblasts. Hence the positive staining of osteoblasts for alkaline phosphatase singles them out as bone forming cells. Approximately 50% of the total circulating serum alkaline phophatase is released from the external surface of the OB plasma membrane. Mineralisation of the matrix occurs mainly in the Haversian systems. OBs also contain receptors for parathormone (PTH) and 1, 25-dihydroxyvitamin D (1, 25 $(OH)_2D$) [67].

OCLs derive from a different lineage to that of the OB. OCLs come from the granulocyte-macrophage colony forming unit (GM-CFU) within the haemopoietic progenitor cell compartment. They are large multinucleated cells which line the surface of cortical or trabecular bone. The most remarkable morphological feature of OCLs is the ruffled border, a complex system of finger- shaped flat plate-shaped projections of the membrane, the function of which is to mediate the resorption of the

calcified matrix. The protein components of this matrix, mainly collagen, are degraded by matrix metalloproteinases and cathepsins O, K, B and L, secreted by the OCL into the area of bone resorption [87]. This digestion of the matrix is the first step towards the resorption of calcified bone. Resorption of bone takes place in Howship's lacunae which are scalloped spaces, where osteoclasts are attached through a specific $\alpha v\beta 3$ integrin to components of the bone matrix such as osteopontin. The resorbing end of the OCL forms a ruffled border which is folded and in contact with bone. Normally the rate the amount of bone formed equals the amount resorbed. The remodelling rate per year for the skeleton is between 2 and 10%. Adult bone remodelling is responsible for the complete renewal of the skeleton every 10 years. The basic multicellular unit (BMU) comprises the juxtaposed osteoclasts and osteoblasts. The average life span of the BMU is 6 months with both OCLs and OBs undergoing apoptosis [67]. The functional co-operation of both cell types in the BMU is critical for the maintenance of bone homeostasis.

Newly formed bone in children or adults has a relatively high ratio of cells to matrix, and is characterised by coarse fibre bundles that are randomly interspaced and interlaced. This type of bone is called woven bone. Lamellar bone is more mature characterised by fibre bundles organised in parallel or concentric sheets. In long bones the concentric arrangement of lamellar bone around blood vessels forms the Haversian systems. Growth in length is dependent on proliferation of cartilage cells and on the endochondral sequence at the growth plate. Growth in width however, is dependent on formation of bone at the periosteal surface and by resorption at the endosteal surface. In adults, after the epiphyses close, growth in length ceases, but remodelling continues throughout life and is influenced by the mechanical stresses it suffers. In addition if injuries such as fracture disrupt the organisation of tissue, progenitor stromal cells can differentiate into cells with functional capabilities different to those

of OBs [88, 89]. This means varying amounts of fibrous tissue and cartilage can be formed. Expanding lesions such as tumours in bone induce resorption at the surface in contact with the tumour [90].

Several factors control bone resorption at both a local and systemic level. The hormones PTH, thyroxine, growth hormone and 1,25 dihydroxy vitamin D_3 stimulate bone resorption and calcitonin, oestrogen and glucocorticoids decrease resorption. PTH mediated resorption works through signals influenced by the OB as these cells express the PTH receptor [91]. The pro-inflammatory cytokines IL-6, TNF, IL-1 are extremely potent stimulators of osteoclast formation and bone resorption.[66]. IL-6 is produced at high levels by osteoblasts in response to TGF-B, PDGF, IL-1 and TNF [92-94].

Growth factors such as Platelet Derived Growth Factor (PDGF) [95], insulin-like growth Factors (IGF) [96], Fibroblast Growth Factor (FGF) [97] and members of the Transforming Growth Factor β (TGF β) superfamily stimulate osteoblast development [95]. They are produced not only by mesenchymal cells but also from the bone matrix particularly when osteoclasts are being resorbed hence the continual homeostatic relationship between bone forming and bone producing cells [98]. TGF β particularly demonstrates this dual ability [99]. It stimulates osteoblast differentiation and bone resorption by stimulating osteoclast formation. Other members of the TGF β superfamily include the bone morphogenetic proteins (BMPs). Type 2 and 4 are able to initiate osteoblastogenesis from uncommitted progenitor cells in vivo [88].

Adhesion molecules such as integrins, intercellular adhesion molecules (CAMs), selectins, cadherins, mucins and leucine rich glycoproteins are involved in both OB and OCL migration from the bone marrow and development [100, 101].

Bone formation is an orderly process in which inorganic material is deposited in relation to an organic matrix. The concentration of calcium and phosphorus in the plasma and ECF influences the rate at which mineral is formed [7, 66]. There is a limit for the concentration of calcium and phosphorus below which mineralisation will not occur. When bone is resorbed calcium and phosphorus ions are released into the ECF and the organic matrix is then resorbed. The calcium in the ECF is remarkably constant. It exists in three forms, as free ions, bound to plasma proteins and as diffusible complexes. The concentration of free calcium is under tight hormonal control as it influences a variety of functions. PTH is the principal hormone which acts directly on bone and kidney, and indirectly on the intestine through its effects on the synthesis of 1, $25(OH)_2D$ to increase calcium concentrations. PTH is influenced by the concentration of serum ionised calcium [7, 66].

Precise orchestration of all the signals controlling new bone formation and resorption must occur to keep the skeleton intact. The dysregulation of any of these pathways can lead to numerous bone diseases e.g. Paget's disease which is characterised by excessive resorption of bone with the haphazard chaotic new bone formation and the risk of malignant transformation.

Paget's disease was first described in 1877 and is a disorder of excessive but disordered bone remodelling [102]. It is predominantly a disease of the elderly and 60% are male. The rate of bone remodelling may be enhanced up to twenty times that of normal turnover rate. In addition, there is both an increase in osteoclastic bone resorption and new bone formation but the normal coupling between bone resorption and formation is imperfect. The bone formation is excessive, irregular and chaotic

[103]. There is a mixture of woven and lamellar bone. The OBs and fibroblasts in Paget's disease are morphologically normal, but the osteoclast is large and has a characteristic phenotype. There is also increased vascular fibrous tissue throughout the marrow and the resulting increased vascularity of the bone is associated with arterio-venous shunting and enhanced local blood flow through the bone. This may cause local warmth over the bones and a high cardiac output state. The most commonly affected bones are the skull, vertebrae, pelvis, axial skeleton and proximal ends of long bones and tibia. Studies by Roodman et al [104, 105] first hypothesised that the bone marrow microenvironment in which the osteoclast forms may be abnormal in patients with Paget's disease. In part this may be explained by the production of IL-6 by the Pagetic osteoclast. Biphosphonates which work by inhibiting osteoclast activity are extremely effective in the treatment of Paget's disease. Roodman also postulated that in addition to IL-6, Receptor activated NF-kappa B ligand (RANKL) is involved as a factor. He postulated that in the case of Paget's disease there is increased RANKL and IL-6 production, and IL-6 enhances the responsitivity of the OCL precursors to RANKL, contributing to the elevated numbers of OCLs. Interestingly in patients with multiple myeloma, a malignancy with uncontrolled monoclonal expansion of plasma cells where 95% to 100% of patients develop bone lesions, both IL-6 and RANKL levels are increased [105].

One of the means whereby osteoporosis can result is through increased OCL formation or activity; osteoporotic patients have thin normally mineralised bone and risk fracturing [106]. Osteopetrosis is a bone disease caused by the impaired osteoclastic bone resorption and is characterised by an increase in normal bone mass which impairs normal bone marrow function [106]. The impaired resorption mechanisms can be due

to either decreased osteoclast formation or decreased capacity of the preformed osteoclasts to resorb bone [107].

The pubertal growth spurt and subsequent cessation of growth are induced by sex hormones [108]. At the end of puberty, high levels of testosterone and oestrogen induce hypertrophy of the remaining epiphyseal plate cartilage. These cartilage cells then die and are replaced by bone. Without any further cartilage formation the growth of these bones ceases. This is known as growth plate closure. Thyroid and parathyroid hormones are also important in regulating the maturation and hypertrophy programme of the epiphyseal growth plate.

Throughout life the skeleton remains a dynamic organ perpetually remodelling itself in response to mechanical stress, autocrine, paracrine and hormonal factors [66]. It seems likely that the development of disregulated growth which could predispose to the development of OS could arise from an abnormality in any of these pathways. Continued stress on bones throughout life can result in "microcracking". Studies have been undertaken in breeds of large or giant dog breeds who are known to be at increased risk of developing canine OS [109], to determine if it is an increase in micro-cracking and remodelling which predisposes to these dogs developing OS. The conclusions have been that microdamage and remodelling are unlikely to be important in the induction of OS [110].

The hormonal influence at the time of puberty when there is a peak in incidence of OS [111, 112] suggests that the multitude of pathways switched on at that time may hold the clue to further elucidate the aetiology of this rare, but very serious disease.

1.3 Osteosarcoma

Osteosarcoma (OS) is a high grade malignant spindle cell tumour which develops within bone. It derives from the osteoblastic lineage therefore distinguishes itself from other primary bone tumours by producing neoplastic osteoid or immature bone. It is a generic term encompassing a number of histological subtypes [113, 114] (Table 1). This thesis will concentrate on the biological and clinical characteristics of classic or central OS.

Conventional (intramedullary osteosarcoma	**Cortex-associated osteosarcoma**	**Disease-associated osteosarcoma**	**Miscellaneous**
Osteoblastic	Parosteal	Osteosarcoma in Paget's disease	Osteosarcoma of the gnathic bones
Chondroblastic	Dedifferentiated parosteal	Osteosarcoma in fibrous dysplasia	
Fibroblastic	Periosteal	Osteosarcomas in Mazabraud's disease	
Epithelioid	High-grade Surface	Post-irradiation osteosarcoma	
Giant-cell rich	Intracortical		
Small cell			
Telangiectatic			

Table 1 - The different histological subtypes of Osteosarcoma

1.3.1 Epidemiology

High grade central OS occurs most commonly in the second decade of life [115]. It has a bimodal age distribution with the second peak affecting older persons particularly if there is a predisposing cause such as Paget's disease [116] or a secondary OS developing in previously irradiated bone [117-119]. It is slightly more common in males than in females, approximately M: F = 1.5:1 and its peak frequency in the growing adolescent follows the pubertal growth spurts for males and females respectively [112]. Epidemiological findings suggest that the adolescent peak in incidence is associated with the pattern of childhood skeletal growth [111]. During puberty girls grow faster than boys but their growth stops earlier [120]. OS rates for girls up to the age of 13 yrs are about 30% higher than they are for boys of that age. By middle to late teens boys are considerably taller and in the 15 to 24 year old age range the rate of OS in males exceeds the female rate by 140% [121]. Although osteosarcoma is the most common primary malignant bone tumour, it remains a rare tumour and only 130 and 1000 new cases are diagnosed each year in the UK. and United States respectively [122, 123]

1.3.2 Biology and pathogenesis of Osteosarcoma

Despite numerous studies trying to construct a model of tumourigenesis for this disease little is known to truly understand its aetiology. OS is thought to be a tumour of osteoprogenitor cells. These are multipotential, hormone-responsive stromal cells in the periosteum and bone marrow, which are capable of differentiating into many lineages depending on their environmental cytokines [124]. Bone marrow stromal cells are the stem cells of skeletal tissue. Commitment and differentiation are usually seen as a unidirectional and terminal process. However, the single cells of the stromal

system maintain a "plasticity" or reversible commitment until very late in the process [125]. Osteosarcomas are often characterised by an excessive production of pathological osteoid by malignant tumour cells. Factors which influence osteoblast development may play a role in the development of OS. It is also known that OS can arise in patients with Paget's disease of the bone [116], [126] and [127]. The abnormal lesions that occur in fibrous dysplasia (the persistence of woven bone where lamellar bone would normally develop) bear a close resemblance to the lesions produced by transgenic mice over-expressing C-*FOS* [128, 129]. Transgenic mice over expressing C-*FOS* have abnormal bone remodelling and osteosarcomas develop in some [130]. The increased expression of C-*FOS* mRNA in lesions caused by fibrous dysplasia suggests that this over-expression may represent the first step in the multistep carcinogenesis of bone sarcomas [131, 132].

1.3.2.1 Chromosomal abnormalities

OS is thought to fall into the class of sarcoma where complex unbalanced karyotypes and alterations of the p53 and retinoblastoma (Rb) pathways occur in most cases. This contrasts with the Ewing's sarcoma family of tumours (ESFT) which have balanced karyotypes such as reciprocal translocations e.g. t (11:22) [133, 134]. In ESFT alterations of tumour suppressor gene pathways are very rare. Cytogenetic abnormalities, both structural and numerical, have been described in OS [135]. In one study, Marker chromosomes, extra pieces of structurally abnormal chromosomes of unidentified origin, were found in 58% of the OS samples [135]. Genomic amplification was seen in 30% of samples. The common numerical abnormalities were gains of chromosome 1 and losses affecting chromosomes 6, 9, 10, 13 and 17 [135]. Loss of heterozygosity (LOH) affecting the loci 3q, 13q, 17p and 18q has been reported [136]. 13q and 17p are the loci for the pRb and p53 tumour suppressor genes

the loss of which is thought to have a role in the pathogenesis of OS. However, despite the fact these cytogenetic abnormalities are well described apart from the implication of *pRb* and *p53* the precise role for the other cytogenetic abnormalities is unclear. One hypothesis is these regions may represent the loci of other tumour suppressor genes important in OS tumourigenesis [137].

1.3.2.2 Tumour suppressor genes

Cancer-causing genetic alterations fall broadly into two functional classes: those that activate cellular genes called oncogenes and those that inactivate cellular genes known as tumour suppressor genes [8]. There is growing evidence that indicates the inactivation of tumour suppressor genes predispose affected individuals to the increased risk of developing cancer. The occurrence of OS in a setting of multiple primary tumours is well recognised but very infrequent [138]. The occurrence of OS and other malignancies is frequently related to treatment, but can also be the result of genetic predisposition. Alterations in *p53, Rb*, and the *MDM2* oncogene are found in about 50% of cases of OS [138, 139]. Germ-line mutations however are extremely rare [140]. The incidence of osteosarcoma is increased up to 500-fold in patients who survive retinoblastoma [141]. The retinoblastoma protein *pRb* is functionally inactivated in most human cancers [142, 143] [144]. Numerous studies in cell culture and animal models suggest that *pRb* has a unique ability to encourage and enforce permanent cell cycle withdrawal, consistent with its role as a tumour suppressor protein. pRb also appears to play a central role in the process of cellular senescence, a tumour suppressive process characterised by proliferative arrest and phenotypic changes [144]. In osteogenesis, loss of *pRb*, but not p107 or p130, blocks late osteoblast differentiation. *pRb* physically interacts with the osteoblast transcription factor, cbfa1[145-147], and associates with osteoblast-specific promoters in vivo in a

cbfa1-dependent fashion. Association of *pRb* with *cbfa1* and promoter sequences results in synergistic transactivation of an osteoblast-specific reporter [145-147]. This transactivation function is lost in tumour-derived *pRb* mutants, underscoring a potential role in tumour suppression. Thus, *pRb* functions as a direct transcriptional co-activator promoting OB differentiation, which may contribute to the targeting of *pRb* in osteosarcoma [148] .

In Li-Fraumeni families who inherit germ line mutations of *p53*, OS is one of the commonest malignancies to develop. A mutation or genetic error in one copy of *p53* makes the cell vulnerable to a second random hit or chromosomal loss. Unremitting cell cycling then compounds DNA mutations, leading to the development of a malignant tumour. The precise molecular events which lead to an OS developing in normal bone are still not clearly defined. Gokgoz et al showed that 22% of all OS samples in their study of 247 patients demonstrated *p53* mutations and that the *p53* status was concordant in all paired primary bone and metastatic samples, suggesting that the *p53* pathway is altered early during OS tumourigenesis [149].

1.3.2.3 Telomerase

Telomeres are nucleoprotein structures that cap the ends of chromosomes, protecting cells from inducing inappropriate DNA damage, from chromosomal loss and recombination. Each time a cell divides the telomeres erode, because of the inability of DNA polymerases to replicate the ends of the DNA. When telomeres become critically short, senescence or apoptosis is induced as normal human somatic cells have a finite proliferative capacity [150, 151]. Cancers on the other hand activate the enzyme telomerase to maintain the telomeres and in so doing maintain their proliferative potential [152, 153]. A minority of cancers employ a different technique

to maintain telomeres: alternative lengthening of telomeres (ALT) is a poorly defined recombination mechanism which is characterised by telomeres which are both longer and more heterogeneous in size compared to cells overexpressing telomerase [25]. OS is different from most cancers in that many OS use the ALT mechanism rather than telomerase to maintain telomeres. OS tumours that are able to express telomerase in their primary samples have been shown to have an increased risk of tumour recurrence and decreased overall survival [154, 155]. This concurs with the growing body of evidence that telomerase expressing tumours or those that employ the alternative telomere maintenance strategy ALT, in other paediatric cancers confers a worse outcome [25].

1.3.2.4 Viral pathogenesis

SV40 DNA sequences have been detected in several human malignancies, including mesothelioma, ependymoma, choroid plexus tumours, osteosarcoma and non-Hodgkin's lymphoma [156-158]. SV40 is a DNA polyomavirus [159]. From 1955 to 1963 the propagation of poliovirus in monkey kidney cells led to the inadvertent contamination of poliovirus vaccines with simian virus 40 (SV40). To clarify whether SV40 infection increases risk of the above named cancers, or of cancers arising in children: cancer incidence in three Danish birth cohorts was followed. After 69.5 million person-years of follow-up, individuals exposed to SV40-contaminated poliovirus vaccine as infants or children had lower overall cancer risk than unexposed individuals. Specifically, SV40 exposure was not associated with increased incidence of any of the associated cancers [160]. The finding of SV40 DNA in tumours from individuals who were too young to have been exposed to SV40-contaminated vaccines led to the suggestion that SV40 was a prevalent transmissible human pathogen. Several studies have now screened human sera for antibodies to SV40 using direct and

competitive enzyme-linked immunosorbent assays (ELISAs). It was found that autoantibodies such as those to BKV and/or JCV cross-react with SV40 and give a false positive test result [161]. It has long been postulated that SV40 large T antigen may inactivate wild-type p53 in the osteoblasts of susceptible individuals, but to date there is no data to support the wisdom that contamination with SV40 predisposes to OS or other cancers [157, 158]. To date there has been no convincing data linking viruses as a major aetiological factor in OS [134].

1.3.2.5 Apoptotic pathways

The *FAS* cell death pathway has been implicated in the chemosensitivity and metastatic behaviour of a number of cancers including OS [162]. FAS ligand (FasL) is a type 1 transmembrane protein that induces apoptosis in susceptible cells by interacting with its receptor, *FAS*, a member of the tumour necrosis receptor family. *FAS* is expressed in many different cells and tissues and is functional outside the immune system [163]. Cancer cells will undergo apoptosis when FASL is presented, unless a mechanism of resistance is present. Developing resistance to this pathway is one mechanism that is commonly exploited by tumours to acquire metastatic potential. Examples of such mechanisms include the down-regulation of *FAS, FASL or FLICE* (pro-caspase 8) or up-regulation of apoptotic inhibitory proteins such as BCL-*2* or FLIP. Some tumour cells express *FASL* on their cell surface enabling a "counter attack" against activated T cells to escape surveillance [164]. The ESFT have been shown to carry the membrane and soluble forms of *FASL* [165] and in certain circumstances ESFT avoid apoptosis by down-regulating surface *FASL* and *FAS*. *FAS* signalling may be implicated in OS metastases [166]. There is a variant of the OS cell line Saos-2, Saos-LM6, where a lower level of *FAS* is expressed compared to its parent Saos-2. *FAS*-transfected Saos-LM6 cells and Saos-2 LM6 control cells were injected into mice. Metastases developed in the mice injected with the control cells

but not in the Saos 2 LM6 cells transfected with *FAS*. This infers that *FAS* may be important to the metastatic potential of OS [167]. Interleukin-12 (IL-12) is known to up-regulate FAS expression. This up-regulation of FAS by IL-12 was also demonstrated in human etoposide-resistant MDA-MB-231 breast cancer cells [168]. In this study it was hypothesised that expression of *FAS* may play a role in the elimination of metastatic tumour cells in the lung, an organ in which FAS ligand is expressed. The antitumour activity of IL-12 may be secondary in part to its ability to up-regulate *FAS* expression on tumour cells, which subsequently increases immune-mediated destruction of osteosarcoma cells. The clinical interest of these findings is to use agents such as ifosfamide which may up-regulate *FASL* expression on OS tumour cells and Muramyl tripeptide phophatidylethanolamine (MTP-PE) which can induce IL-12 in patients through the activation of macrophages. This combined induction of both IL-12 and *FasL* could lead to increased tumour cell kill [169]. Duan et al demonstrated that treatment of OS cells with 4-hydroperoxycyclophoshamide (4-HC), the active metabolite of cyclophosphamide, led to an increase in *FASL* mRNA and protein expression. In contrast, 4-hydroperoxydide-chloroclophosphamide (4-HDC), the inactive metabolite of cyclophosphamide, Adriamycin, methotrexate and cisplatin all failed to induce FASL expression.[170]. The proposed idea that there is a cell specific response to various chemotherapeutic agents with respect to the up-regulation of FasL may be the mechanism which may also explain the clinical results of a phase III trial in which patients treated with both ifosfamide and MTP-PE compared with either individual agent had a trend toward improved outcome [171].

1.3.2.6 Other aetiological factors

Osteosarcoma, like other cancers have a diverse range of subtypes and biological behaviour. Known clues which allude to its aetiology stem from the fact it has its highest incidence around the time of increased skeletal growth at puberty which

follows a sex specific time course, and that it most commonly affects the metaphysis of long bones. This period is also marked by the highest levels of circulating physiological growth hormone as well as high levels of Insulin-Like Growth Factor 1 (IGF-1). The prevalence of OS is greatest in dog breeds with the highest levels of circulating IGF-1 concentrations [172, 173]. In one retrospective study of 37 patients, circulating levels of IGF-1 and IGFBP-3 were not predictive of the development of OS [174]. Osteosarcoma occurs in rapidly growing bones that undergo the most stress of weight bearing, with the sites about the knee (distal femur and proximal tibia) being the most common. The role of weight-bearing trauma as a possible aetiological factor is suggested by the fact that osteosarcoma is 80 times more common in large dogs weighing over 20lb than in smaller breeds. In addition, the most common site of osteosarcoma in dogs is also the bones that bear the most weight, the forelimbs (c.f. page 41) [110].

1.3.3 Clinical Features

OS may be suspected on the basis of characteristic clinical and radiological features. Bones around the knee joint and the proximal humerus are the most common sites of occurrence. In up to 90% of cases, tumours arise in the long bones with the axial skeleton, hands and feet less commonly involved.

Individuals complain of pain and swelling but their symptoms are often overlooked as "growing pains" (Figure 4). Many individuals will pin point the development of their symptoms to a previous trauma so prolonging the investigation to the true nature of their pain.

Figure 4 - Clinical presentation of a right distal femoral OS

The plain x-ray appearance of OS shows a mixed picture of increased radiodensity due to the tumour's production of new calcified bone and radiolucency where the tumour has not yet ossified (Figure 5). Cortical destruction with periosteal elevation can often be determined on a plain film x-ray. Magnetic resonance imaging (MRI) of the primary lesion needs to be performed to accurately determine intramedullary involvement of the tumour and the degree of extraosseous extent of soft tissue extension. The soft tissue extension in sclerotic lesions is calcified and has a typical "sunburst" appearance on x-ray.

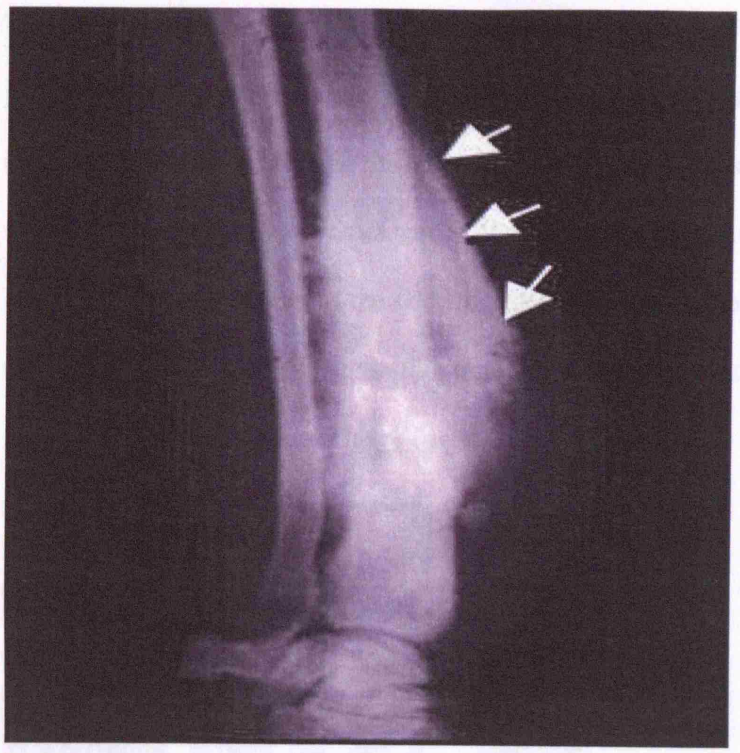


Figure 5 - The plain x-ray appearance of OS with a typical periosteal reaction

The diagnosis is nowadays generally established by core needle biopsy. Detractors of radiological guided percutaneous needle core biopsies felt sufficient material could not be obtained to ensure an accurate diagnosis. However, many studies have been published looking at the diagnostic accuracy of image guided percutaneous biopsies which confirm not only that it is a safe, but an accurate technique for both diagnosis and grade of tumour [175, 176]. Patients who present with the typical radiographic features suggestive of a primary bone tumour should be referred to a sarcoma specialist centre promptly where all aspects of their management can be undertaken. The biopsy should be performed by a sarcoma trained orthopaedic surgeon or interventional radiologist. This is because the biopsy must be planned carefully with consideration to subsequent definitive surgery. A poorly conceived and poorly placed biopsy may jeopardise the subsequent treatment, especially a limb-salvage procedure.

The biopsy sample is placed immediately in a dry sterile container and on reaching the pathology laboratory the tumour is first placed on a sterile microscope slide. The sample is then removed from the slide and placed in a container with formalin for fixation. This cell smear onto a sterile glass slide is known as imprinting. Any loose cells which have fallen onto the slide are fixed and stained for alkaline phophatase. If the imprints of the tumour cells stain positive for alkaline phosphatase, this confirms it is a bone forming tumour (Figure 6).

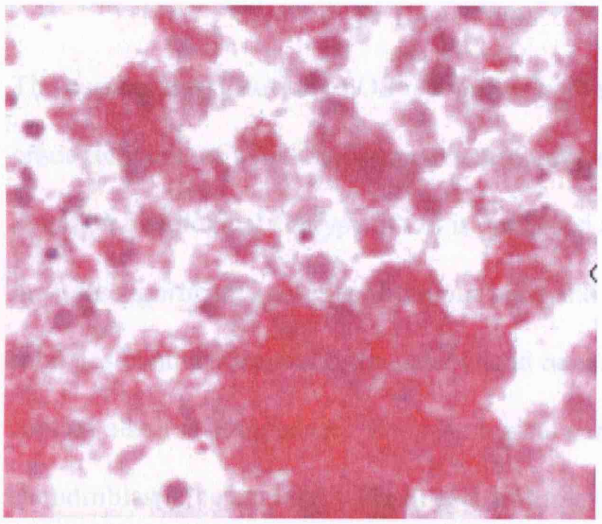


Figure 6 - Alkaline Phophatase imprinted slide

Histological confirmation is essential to differentiate benign from malignant lesions as well as accurate subtyping, diagnosis and grading. Benign bone forming lesions such as osteochondromas and osteoblastomas can also have positive alkaline phophatase imprints. Currently, most tumours are categorised on the basis of morphology. A typical appearance of an osteosarcoma when stained with Haematoxylin & Eosin (H&E) is illustrated in Figure 7.

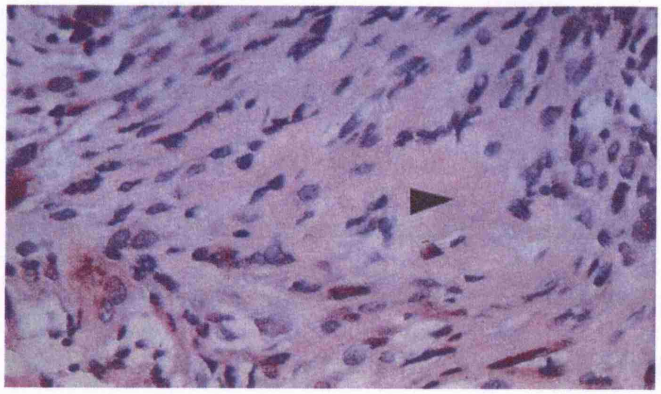


Figure 7 - The Haematoxylin and Eosin staining of OS

The current classification of OS recognises high grade, intermediate-grade and low grade tumours, of which high grade central tumours are the most common. The most common histological subtype of OS is described as "mixed" or "common" and includes significant amounts of tumour bone and osteoid. Other less common subtypes contain minimal or no osteoid and bone. In all subtypes the tumour cells contain alkaline phophatase. The histological subtypes are common/mixed, chondroblastic, telangietatic, small cell, fibroblastic, osteoclast rich, osteoblastic, epitheloid and anaplastic [177]. An analysis by Hauben et al [178] looked at data on 570 patients with biopsy proven localised primary central OS. The primary aims of this study were firstly to investigate whether the histological subtype in the biopsy sample matched the subtype of the OS in the resected specimen, and secondly to see if the subtype influenced either histological response to chemotherapy or overall survival. In comparison with the common/mixed subtype, there were a higher proportion of good responses in the fibroblastic group and a lower proportion of good responders in the chondroblastic group. Patients whose tumours underwent greater than 90% necrosis in response to cytotoxic chemotherapy damage where classed as "good responses". Conversely any tumour which did not undergo this degree of cell death i.e. less than 90% necrosis, were classed as "poor responders". Those patients

who had a "good" response following chemotherapy had significantly better survival than those whose tumours responded poorly to pre-operative chemotherapy ($p<0.01$) (Figure 8). Overall survival did not significantly differ within the subtypes. To date, no other reliable marker has been identified as having independent prognostic information for this group of patients. This means all patients who present with primary OS are offered neoadjuvant chemotherapy with the most active drugs in this disease. These regimens include combinations of Cisplatin, Methotrexate, Ifosfamide and Doxorubicin before the tumours are resected. This entails at least 6 to 9 weeks of cytotoxic treatment with all its inherent toxicities both acute and long term which may not benefit all individuals.

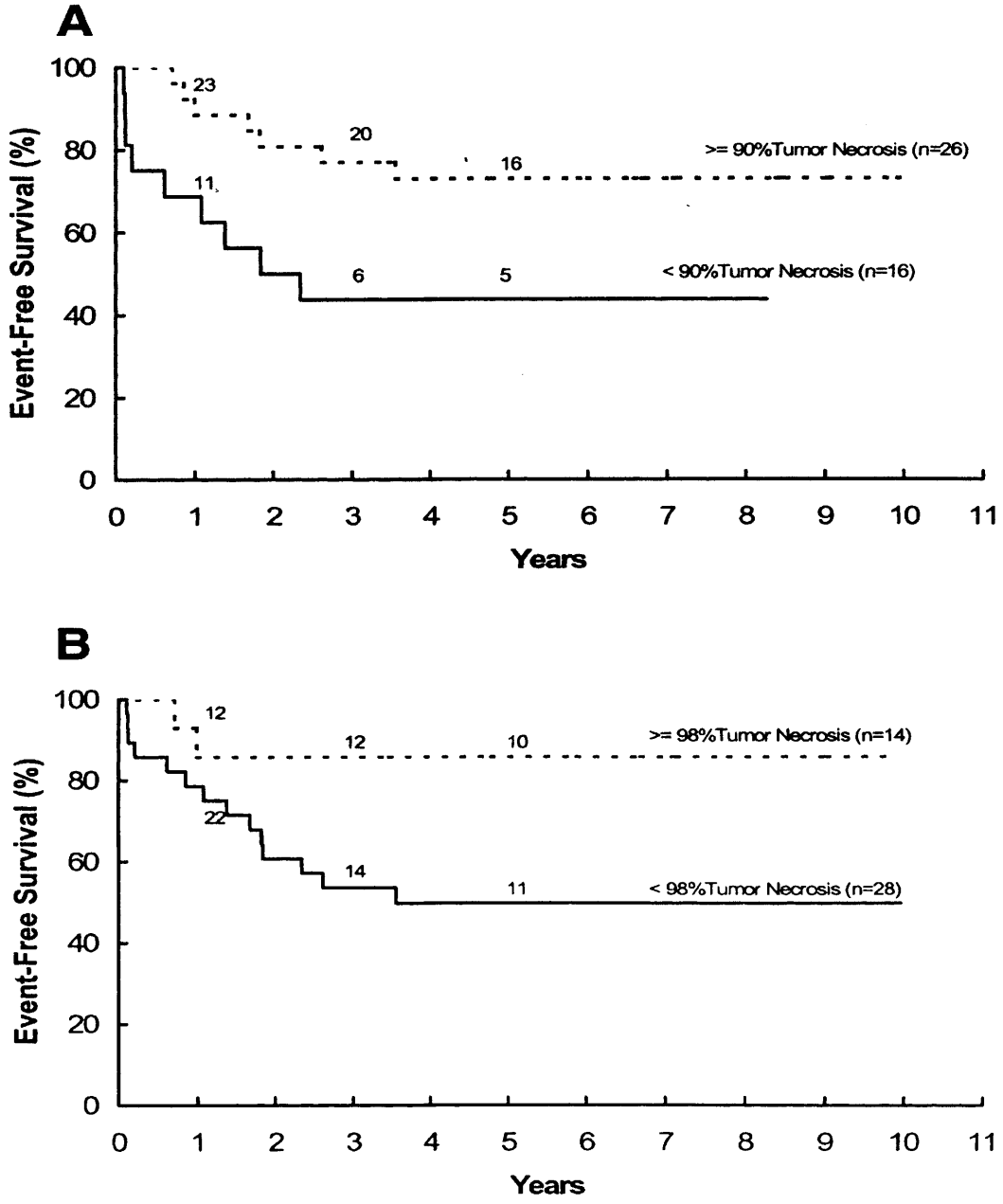


Figure 8 - Survival comparing pathological response

Survival comparing pathologic response at (A) less than 10%residual viable tumour versus [>=] 10% residual viable tumour and (B) less than 2% residual viable tumour versus [>=] 2% residual viable tumour (from Goorin, A. M. et al. J Clin Oncol; 21:1574-1580 2003)

Clinical staging consists of a computerised tomogram (CT) scan of the lungs and an isotope bone scan. In 90% of cases the lung is the initial site for metastatic disease. Bone metastases occur in about 10% of cases and can occur in the absence of pulmonary disease. Simultaneous lung and bone metastases occur in 2% of all cases [179]. A bone scan can detect metastases including "skip" lesions which occur in the same bone as the primary lesion. Since the introduction of chemotherapy, more uncommon sites of metastases have been described e.g. brain secondaries, particularly in those who relapse late.

1.3.4 Management

The management of individuals in whom a diagnosis of OS is made requires the expertise of a multidisciplinary team which includes the surgeon, radiologist, pathologist, oncologist, physiotherapist and specialist nurses. Up until the 1970's the outlook for a patient with OS was miserable. Following amputation for extremity disease there remained only a 10-15% rate of 5-year survival. About 50% of patients would relapse and die following the development of secondary disease in their lungs within six months of surgery for their primary tumour. With this high rate of relapse despite apparently curative surgery the inescapable conclusion was that these patients clearly had micrometastatic disease at the time of presentation. This led researchers to initiate trials of adjuvant chemotherapy in the treatment of OS given the success of adjuvant chemotherapy in other childhood cancers such as Wilms tumour and Rhabdomyosarcoma. The first trials undertaken in the early 1970's did demonstrate a relapse free survival of 45 – 60% at three to five years but these studies were conducted without concurrent control groups [180-182]. In June 1982 the Multi-Institutional Osteosarcoma Group launched a randomised control trial. The primary objective of this trial was to determine whether the administration of intensive, multiagent chemotherapy as adjuvant treatment after definitive surgery of the primary

tumour would significantly improve disease-free and overall survival among patient with non-metastatic OS of the extremity compared with controls treated with surgery alone. Although 113 eligible patients were identified in this study period only 36 accepted randomisation such was the controversy surrounding the management of OS at that time. The results showed a favourable relapse free survival in the region of 66% for those receiving adjuvant chemotherapy, similar to the magnitude gained in the previous uncontrolled studies. The follow up period was too short to demonstrate an overall survival gain [183]. A second randomised controlled study which began recruiting a year earlier in 1981 randomised 59 patients to surgery alone or surgery followed by adjuvant chemotherapy [184]. Both studies used several agents including high–dose methotrexate, adraimycin and actinomycin D. This study was discontinued in 1984 when it became apparent that there was a statistically significant advantage for those receiving adjuvant chemotherapy. Both these studies demonstrated that adjuvant chemotherapy unequivocally benefits patients with primary non-metastatic extremity OS.

About 10-20% of patients however will present with clinically detectable metastatic disease at initial diagnosis. Whilst their outcome is less favourable than those who present with localised disease the introduction of aggressive therapeutic approaches such as the simultaneous resection of both primary and amenable metastases can improve the survival of selected individuals [185].

Once a diagnosis has been established and full staging has been performed the MDT must recommend treatment decisions based on whether the patient has presented with non-metastatic extremity disease, synchronous primary and secondary disease or relapsed disease.

1.3.4.1 Surgical management

For those that present with non-metastatic primary extremity OS the aims of treatment are to control their primary disease and treat occult micrometastases simultaneously. Until the 1980's the standard surgical management for extremity osteosarcoma was amputation. However since the introduction of neoadjuvant chemotherapeutic regimens and new surgical techniques limb salvage surgery is a consideration for all patients. The choice between amputation and limb-sparing resection must be made by an experienced orthopaedic oncologist in the setting of the MDT, taking into account tumour location, size, and extramedullary extent; the presence or absence of distant metastatic disease; and patient factors such as age, skeletal development, and lifestyle preference. The most recent comparison of the results of limb-sparing surgery and amputation were reported by Sluga [186] and colleagues from the University of Vienna, evaluated 130 consecutive patients younger than 21 years of age treated for osteosarcoma of the extremity. Ninety percent (116 patients) were treated by a limb-sparing procedure. Fourteen amputations, 32 rotationplasties, and 84 resections with subsequent reconstruction were performed. The 5-year metastasis-free survival rate was 60% for patients treated by amputation or rotationplasty and 71% for patients treated by limb-sparing surgery. The surgical margins were classified as wide in 109 cases and radical in ten cases. The overall local recurrence rate was 2.3%: 4.3% for amputation and/or rotationplasty and 1.2% for limb-sparing surgery. The overall survival rate was significantly influenced by tumour volume (P = .0018), response to chemotherapy (P = .039), and presence of metastases at the time of diagnosis (P = .0001). The authors warn that limb-sparing is not suitable for every patient; patients with large tumours and close margins may require amputation. They emphasise the importance of wide margins to a successful limb-sparing procedure. This study, as well as previous studies, showed no disadvantage in patient survival, local recurrence

or Quality of Life (QoL) for patients treated by a limb-sparing procedure and those undergoing an amputation. In the UK for approximately 8-10% of patients presenting with osteosarcoma amputation is the treatment of choice. It is now usually reserved for those who have gross neurovascular or skin involvement. Rougraff and colleagues [187] evaluated 227 patients with non-metastatic osteosarcoma of the distal femur treated at 26 institutions. They reported eight (11%) local recurrences in 73 patients with a limb salvage procedure, and nine (8%) local recurrences in 115 patients who had an above-knee amputation. No local recurrences were reported in the 39 patients who had a hip disarticulation. The safety and efficacy of limb-sparing procedures for high-grade bone sarcomas is well proven and remains the treatment of choice in all eligible patients.

1.3.4.2 Systemic chemotherapy

The use of neoadjuvant chemotherapy for osteosarcoma increased in popularity in the late 1970's. This is the giving of upfront systemic treatment for localised disease. It was as a direct consequence of orthopaedic surgeons introducing the concept of limb-savage surgery for extremity disease. It was initially given shortly after biopsy, staging and diagnosis in an effort to utilise the time effectively whilst the custom built prosthesis was being made. The making of a prosthesis could take up to 2-3 months so it was unacceptable to leave a patient with such a high grade tumour with its potential for further growth and metastasis without definite treatment. The pre-operative time lag was taken up with the patients receiving a regimen of cytotoxic treatment containing a combination of the most active drugs used in osteosarcoma. When the first trials of this neoadjuvant approach were analysed retrospectively it appeared that the patients that underwent pre-operative chemotherapy did better than the patients who underwent immediate surgery and post operative chemotherapy, the classical adjuvant approach [188, 189]. In addition investigators noted that they now had a

powerful new prognostic tool when measuring the histological response of the tumour to chemotherapy. Patients who undergo pre-surgical chemotherapy have their resected tumour assessed for chemotherapy damage; this is measured by the degree of induced necrosis. A number of grading systems have been developed. The original Huvos model served as a template for other grading systems [190, 191]. The degree of necrosis induced by the chemotherapeutic agents was graded. If it fell short of 90% these patients were deemed as unfavourable responders. The outlook of the unfavourable responders was poor. This prognostic value has been confirmed in multiple clinical trials. The definition remains imprecise. Standard or poor response is now most frequently defined as the persistence of more than 10% viable tumour. Less than 95 or < 98% tumour necrosis have also been employed as standards [192, 193].

Bacci at al at the Rizzoli Institute treated 510 patients with extremity osteosarcoma between March 1983 and June 1995 with different regimens of neoadjuvant chemotherapy. The factors that influenced the histological response were investigated. The rate of total necrosis was not related to the patients' gender, age, site, size of tumour, serum of alkaline phosphatase values, or route of cisplatin administration. The histological response significantly and independently correlated with the number of drugs administered before surgery and with the histological subtype of the tumour. According to the number of drugs used, the percentage of total necrosis was 31% for a four-drug regimen, 18% for a three-drug regimen, and only 1.5% for a two-drug regimen. According to the histological type, the rates of total necrosis were 41% for telangiectatic tumours, 36% for fibroblastic tumours, 15% for osteoblastic tumours, and 3% for chondroblastic tumours [194, 195].

Those patients who demonstrate a poor histological response are deemed as high risk and are more likely to develop metastatic disease. Neoadjuvant chemotherapy

provides treating physicians with the most powerful prognostic tool. By measuring the amount of viable tissue and degree of necrosis induced by cytotoxic chemotherapy in a resected sample, an accurate prediction can be made about the risk of relapse (See Figure 1.7 page 55). In addition it also offers the theoretical advantage of treating micrometastatic disease upfront. The range of drugs active in the management of osteosarcoma is limited. Highest single agent response rates are seen with cisplatin, doxorubicin, high dose methotrexate and the alkylating agents' ifosfamide and cyclophosphamide. To improve overall response rates combinations of drugs were introduced. There has been no doubt the introduction of combination chemotherapy in the early 70's improved the overall survival rates of patients with high-grade extremity osteosarcoma from 10-15% to 50-60%. The case however for neoadjuvant chemotherapy being superior to immediate surgery and adjuvant treatment in terms of overall survival has yet to be proven. The initial encouraging retrospective data from Memorial Sloan Kettering has been tempered not only by other studies but also by their own update. The ultimate advantage neoadjuvant chemotherapy should have over adjuvant therapy is that it should improve overall survival. The drug resistance model of Goldie [193] suggests that as long as management of the local disease is not compromised, early systemic chemotherapy should yield superior results compared to delayed post-operative chemotherapy. Goorin et al recently published a Paediatric Oncology Group (POG) study where 106 patients with high grade non-metastatic OS were enrolled between 1986 and 1993 and randomly assigned to immediate surgery or immediate chemotherapy. Those assigned to immediate surgery received chemotherapy post-operatively. They concluded chemotherapy was effective in both treatment groups but there was no advantage for event free survival (EFS) for patients given neoadjuvant chemotherapy over adjuvant therapy [196]. If it cannot improve survival in the neoadjuvant setting then at least pre-operative chemotherapy could

provide an in vivo model of drug effect on the tumour. Perhaps poor responders to one regimen should be able to undergo salvage chemotherapy post-operatively in an effort to improve their long-term outlook. This approach has been undertaken by a number of institutions but to date there is little evidence that changing chemotherapy agents in those poor responders improves their long term [197]. The Rizzoli group have recently published an updated report for the long-term outcome of patients with non-metastatic osteosarcoma who had their treatment changed if their tumour had a poor pathological response. The median follow up was 11.5 years and they demonstrate no difference in prognosis in good versus bad initial responders when the bad responders were switched to post operative ifosfamide and etoposide [198]. The cornerstone of the European Osteosarcoma Intergroup (EOI) trials has been a two drug regimen consisting of six cycles of cisplatin and infusional doxorubicin. Although the two drug regimen has never been shown to be inferior to other treatments [199, 200] many clinicians feel uneasy using regimens without ifosfamide and methotrexate as several centres have clearly demonstrated superior results [201-203]. For this reason a collaboration between four major research groups in OS: the North America Children's Oncology Group (COG), the German-Austrian-Swiss Osteosarcoma Intergroup (COSS), the European Osteosarcoma Intergroup (EOI) and the Scandinavian Sarcoma Group (SSG) has been formed and a study EURAMOS 1 has been launched to investigate whether it is feasible to improve outcome for both good and poor responders through the addition of extra agents into the post-operative treatment schedule. The control arm drugs include methotrexate, cisplatin and doxorubicin. Poor responders will be randomised between more of the same pre-operative drugs or the addition of ifosfamide and etoposide. Good responders will be randomised to more of the same regimen as pre-operatively or the same drugs plus the addition of maintenance pegylated interferon ά-2b.

It is important to differentiate those patients who are presenting with synchronous primary and metastatic disease from those who have relapsed with metastases months to years after their combination chemotherapy and surgical management of their primary disease. The former group have never been exposed to cytotoxic chemotherapeutic agents whereas that latter are presenting with recurrent disease which reflects the emergence of chemoresistant micrometastases. For patients that present with lung only metastases, surgical resection of the pulmonary lesions with or without chemotherapy can bring about long term survival if successful. Patients that relapse with solitary brain only metastases can also be considered for a cerebral metastastectomy which if completely resected may improve long term survival [204].

Resection of pulmonary nodules for many malignant diseases was based on the successful case of a patient who in 1939 underwent resection of their solitary lung metastasis from a primary renal carcinoma. He enjoyed many years of event free survival [205]. That initial case report describing the successful resection of pulmonary metastases leading to long term disease control has led to this approach being adopted for the suitable patient across a wide number of tumour types. For patients with OS many small series have been published looking at long term outcomes for those who have undergone successful resections [206-208].

The chemo naive patients are less likely to present with chemo-resistant tumours and need control of their primary tumour included in their overall management as well as how to manage the metastatic disease. For this group, surgical resection is more likely to follow chemotherapy. For the group that present with relapsed disease following chemotherapy the role of second line treatment is not clearly established and decisions should be made on an individual basis, taking into account factors such as extent of disease, disease free interval and details of previous chemotherapy. This group not

only has more inherently resistant tumours but also they carry a higher probability of extra-pulmonary recurrence.

Martini et al in 1971 published a series of 22 patients in whom all pulmonary metastases were resected. Only 6 of this series had solitary metastases with the remaining 16 having multiple nodules. This group of patients had 58 thoracotomies between them, some undergoing 6 procedures. What was startling about this series is that metastatectomy had improved the survival, 3 years after amputation for their primary disease from 5% to 45%. This success then led to the approach of aggressively resecting all pulmonary disease in patients in whom the primary treatment was reasonable controlled, the patient was assessed as fit for surgery and there was no alternative treatment for their metastatic disease. In 1984, Goorin et al reported on the follow up of 93 patients treated for non-metastatic osteosarcoma. All patients underwent surgery and adjuvant chemotherapy for their primary disease. 32 of these patients relapsed with only pulmonary disease. By applying a stringent set of surgical criteria, complete resection of pulmonary metastases was achieved in 11 of the 26 who underwent surgery. Nine of the eleven were disease free at the time of reporting with a median survival of 42 months, whereas only 2 of the 15 who underwent incomplete resection were free of disease at 57 and 101 months. He concluded that a significant difference ($p=0.005$) in survival from initial relapse for patients who were completely surgically resected was seen, even when the result was stratified from time to first relapse and number of pulmonary nodules ($p=0.005$) [209]. Since this publication where similar criteria have been adhered to, current studies have verified these conclusions. Ward et al concluded after a review of 111 patients that underwent pulmonary metastastectomy that survival was best in: patients with three or fewer pulmonary nodules who present with synchronous metastatic and primary

disease, those with four or fewer recurrent pulmonary nodules and patients whose pulmonary metastases were confined to one lung. A longer disease free interval between primary tumour resection and the development of metastases is also associated with a more favourable outcome. This study demonstrated a median survival of 60 versus 28 months for patients operated on and those who did not undergo surgical resection of their pulmonary metastases. This range of different scenarios means that each case must be taken on its individual merits. The presence of bilateral disease does not preclude curative resection [210]. Instead they may be approached by staged thoracotomies or median sternotomy. Repeated thoracotomies have also been performed and achieved prolonged survival as long as all macroscopic disease has been removed. 5-year survival rates of 30-40% have been reported for such individuals. Complication rates have been cited in 11.7% of procedures with 0% surgical mortality. The question of whether the addition of chemotherapy can further enhance this need to be addressed in both the pre-surgical and post-surgical setting. Interestingly as chemotherapeutic strategies for primary disease are intensifying with improving survival rates there may be a case that fewer of these patients are salvageable after relapse as they may have chemo-resistant disease.

There is no randomised trial evidence to suggest what the additive role of second line chemotherapy may have in terms of relapse free and overall survival following pulmonary metastastectomy [211, 212]. There is available however single institution data, which correlates clinical outcome to the histological response of the pulmonary disease. The Rizzoli Institute has reported on the clinical course and outcome of 28 patients who presented with synchronous lung metastases at the time of first diagnosis. All patients underwent neoadjuvant chemotherapy followed by synchronous excision of both their primary and metastatic disease. Although they concluded they were a

much more heterogeneous group of patients in terms of prognosis, disease free survival was significantly higher for patients with only one or two metastatic lesions than for patients with three or more lesions. They also found a strong correlation between degree of necrosis of the primary and secondary tumour. Like the case for primary disease where good responders had improved relapse free survival so it appears that those with pulmonary metastases who have a greater than 90% necrotic response will also have improved survival.

Rosen at el reviewing the outcome of their patients treated by pulmonary resection plus or minus "adjuvant" [213] at UCLA suggested the following guidelines for the future management of such patients. Patients who presented with either one or a few similar sized nodules, who had either completed their adjuvant therapy or at least a year had passed since their primary surgery, it was proposed these patients had a thoracotomy to resect all disease followed by chemotherapy. Patients who presented with a few small nodules either synchronously or within 12 months of the diagnosis of their primary tumour, or those patients who presented with multiple nodules of varying sizes at diagnosis should first be treated with chemotherapy followed by thoracotomy. If the nodules of this group of patients shrink or remain the same size after 3-6 months of chemotherapy then they should proceed to thoracotomy. In the absence of trial data these single institution studies suggest that the addition of chemotherapy in chemosensitive disease can improve the overall outcome by not only rendering the patient more operable but by treating further occult metastatic nodules.

1.3.4.3 Drug resistant pathways

The human *MDR1* gene was identified in the mid 80's. Its product P-glycoprotein

is a transmembrane glycoprotein that is normally present in cells of the adrenal cortex, biliary canaliculi, endothelium of the blood-brain and blood-testicle barriers, placenta, gastrointestinal epithelium, proximal renal tubuli and some bone marrow stem cells [214]. In many of these organs P-glycoprotein is thought to act as a detoxifying agent, by pumping toxins out of cells. In the adrenal cortex and placenta however it may transport the steroid sex hormones. Increased levels of P-glycoprotein are found in many cancer cells and increased levels are detected after cytotoxic chemotherapy particularly when the tumour has become refractory to treatment [215]. In OS p-glycoprotein expression has been extensively studied. There are inconsistencies in some of the results of the various studies trying to elucidate its role but the consensus is that P-glycoprotein is a marker of poor outcome and drug resistance.[216]. In this study by Baldini et al however no correlation was demonstrated between the extent of tumour necrosis to cytotoxic chemotherapy and P-glycoprotein status. To date the extent of necrosis following induction chemotherapy is the most powerful prognostic factor available. Does this mean we can infer anything clinically meaningful from either the presence or absence of p-glycoprotein? Does its absence predict for a good response to treatment? Clinical trials of agents such as cyclosporine and verapamil that reverse the multidrug-resistance phenotype in vitro have been disappointing [217]. In OS the commonly used first line drugs include cisplatin, doxorubicin, ifosfamide and methotrexate. Only doxorubicin falls into the type of agent that P-glycoprotein is able to pump out from the cells. The others are not substrates of P-glycoprotein so it may be that poor outcome is not related to decreased drug accumulation but other factors.

1.3.4.4 Summary and future directions

The optimum management of patients who present with non-metastatic OS is the carefully planned surgical removal of the primary tumour plus systemic chemotherapy pre- and post-operatively to eradicate occult micrometastatic disease and so improve long term survival. Unfortunately recurrent disease still occurs in 30-40% of patients who undergo this intensive management. The degree of tumour damage to pre-operative cytotoxic chemotherapy can predict for those who will do less well but currently there are no proven intervention strategies to circumvent this. Improvements in radiological imaging of these tumours pre-operatively [218] may help predict the poor responders but again without alternative treatments this technology cannot change the outlook for such patients. There is an urgent need for the better understanding of the biology of this disease so either predictive or prognostic markers can be identified which would have a role not only for stratifying treatments but also provide new targets for which treatments could be developed.

1.4 Gene Expression Microarray

Cancer arises from a stepwise accumulation of genetic changes which have escaped the normal control pathways which regulate cell proliferation. The cancer phenotype is a complex process. However with the introduction of high throughput technologies such as gene expression microarray (GEM) genomic and proteome research is a new frontier for the molecular characterisation of cancer. By comparing the genes expressed in cancer samples to those expressed in normal cells a cDNA expression pattern unique to that cancer subtype may be obtained. This is termed the molecular fingerprint or signature. From this information the functional status of the cells or tissue may be determined. By uncovering the expression profiles of tumours it is hoped that functionally important molecules will generate information to tailor a

treatment to the individual patient. Microarray technology allows the examination of gene expression patterns using thousands of genes simultaneously. The technique of southern blotting [219-221] developed in the 1970's exploited the fact that DNA is exquisitely specific for its complementary sequence (cDNA) even when present in a complex mixture. Whilst it was a very specific technique it was limited by the fact only one gene could be analysed at any one time making the whole process of identifying involved genes rather laborious and time consuming. The advent of robotics allowed the automated construction of several thousand genes to be spotted on to a single membrane or glass slide so allowing the analyses of several thousand genes in one single experiment. Vast amounts of data are generated then stored and analysed by computers. Whether a glass slide or nylon filter is used thousands of DNA probes of known sequence are spotted in an ordered fashion to their solid phase support. Each spot is complimentary to a specific gene or expressed sequence tag (EST) sequence. The many applications of this new technology include:

- Genotyping
- Study individual's genetic makeup; preventive medicine; diagnosis
- Pharmacogenetics

There are different types of arrays. Pre-made microarrays differ from one type to the next based on a few entities.

- The microarray substrate; nylon or glass
- What is physically applied to the substrate material
- The labelling reaction and reagents
- The hybridisation technique

The surface used for glass arrays is usually coated with positively charged silane or poly-lysine to which negatively charged DNA molecules bind. Microarrays are either

cDNA probes (e.g. Research Genetics™) or oligonucleotides (e.g. Affymetrix). The cDNA is cross-linked to the matrix and denatured using heat or alkali. Nylon filters were used in this work. These were supplied by Research Genetics ® Huntsville, Alabama USA. The DNA spotted onto the human GENEFILTER™ membrane was obtained from the I.M.A.G.E (Integrated Molecular Analysis of Genomes and their Expression) consortium at the Lawrence Livermore National Laboratory (I.M.A.G.E./LLNL cDNA) [222]. Clones were selected from the Unigene [223] database containing the 3' UTR. Each clone is verified by streaking single colony isolation and sequencing. Each sequence is verified against GenBank utilising NCBI BLAST [224]. At least 5μg of verified, inert DNA is spotted onto a positively charged nylon membrane. The DNA is then UV cross-linked to the membrane. A system of controls, including both total human genomic DNA and putative housekeeping genes, is also provided on the membranes. The house keeping genes are selected based on function and hybridisation results across several different tissues. All of the controls can be used for orientation of the membrane and alignment when utilising the Pathways™ software (Research Genetics ® Huntsville, Alabama USA). Glass offers many advantages over nylon filters. It is impermeable and smooth allowing rapid diffusion during hybridisation and washing. Its flatness, rigidity and transparency also improve image acquisition and analysis. The labels used to detect bound cDNA also differ between the various commercially available filters, be they glass or nylon. Probes can be labelled fluorescently or with radionucleotides. Cy3 (green) and Cy5 (red) are the most commonly employed fluorescent labels. Two samples each labelled with a different fluorophore hybridise competitively to the complimentary probes on the microarray. Each of the immobilised probes on the microarray is large enough to accommodate both fluorescently labelled probes. The resulting appearance of the microarray is a measure of the relative amounts of each sequence between the two

samples, thus if the Cy3 labelled probe (green) has bound more the output will be a green spot, if the Cy5 (red) labelled probe has bound more it will result in a red spot and if both probes have hybridises to the same extent the resulting spot will be yellow. Figure 9 summarises the whole process from extraction of RNA, to making the cDNA probe and the resulting array. In this research thesis a separate reference filter was made by hybridising a cDNA labelled probe made from a mixed pool of RNA derived from Human osteoblast cells, peripheral blood mononuclear cells and human endothelial cells (see methods 2.9 pg 92). This allowed comparison of all classical central high grade osteosarcoma samples to "normal bone and stroma" [225].

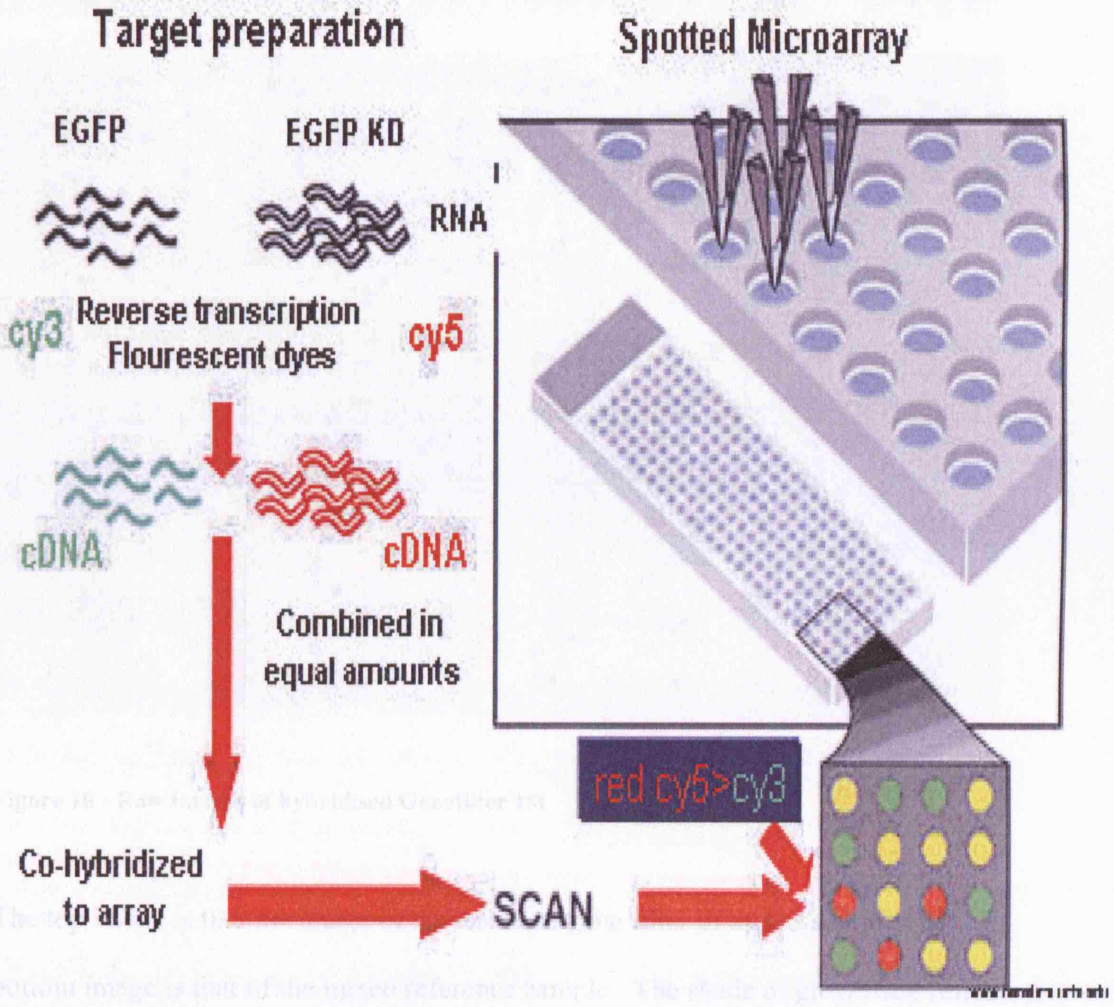


Figure 9 - Schematic outline of microarray data

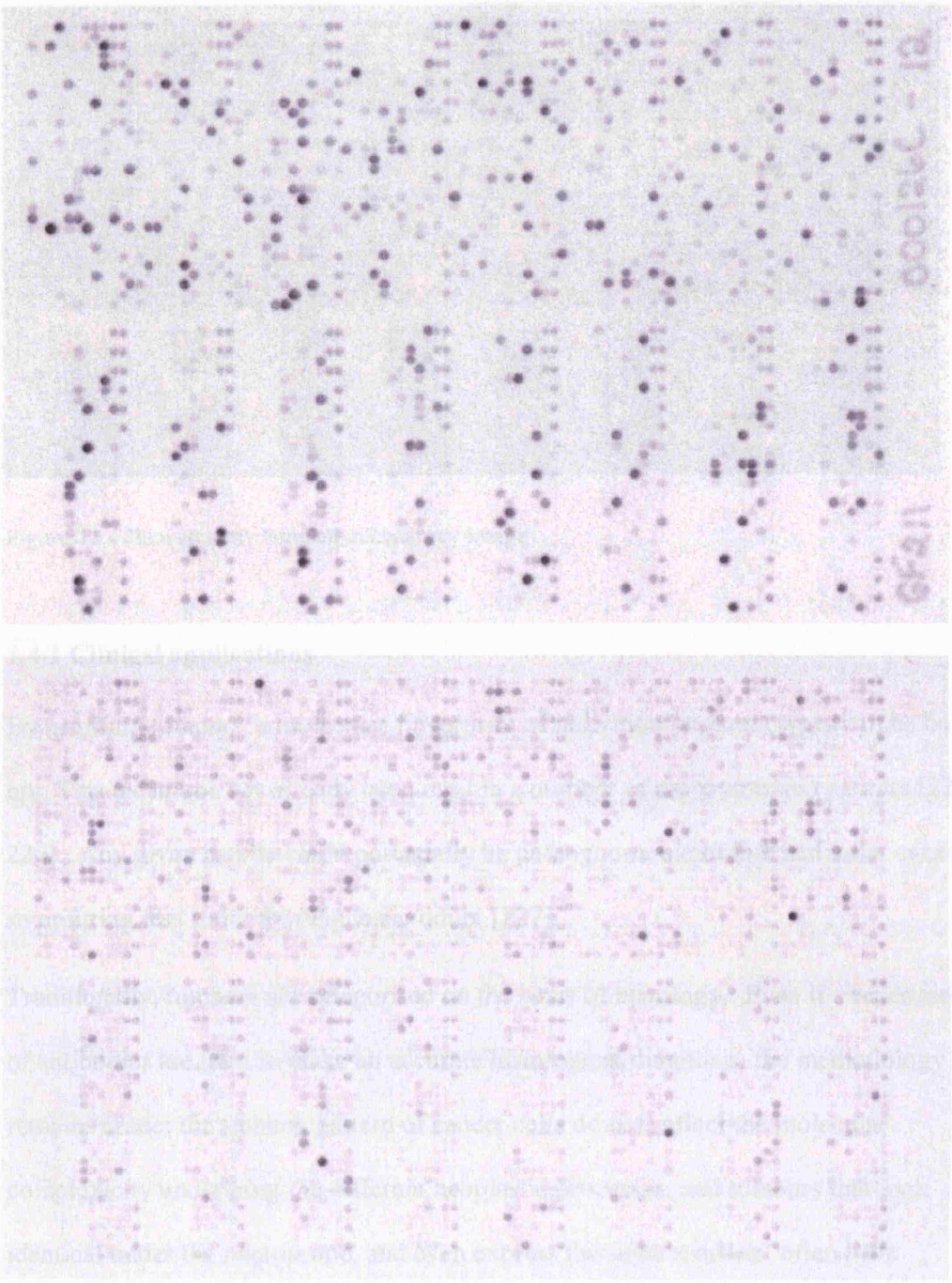


Figure 10 - Raw images of hybridised Genefilter TM

The top image is the raw image of a hybridised gene filter of an OS sample and the bottom image is that of the mixed reference sample. The shade of grey/black reflects the intensity of the hybridisation.

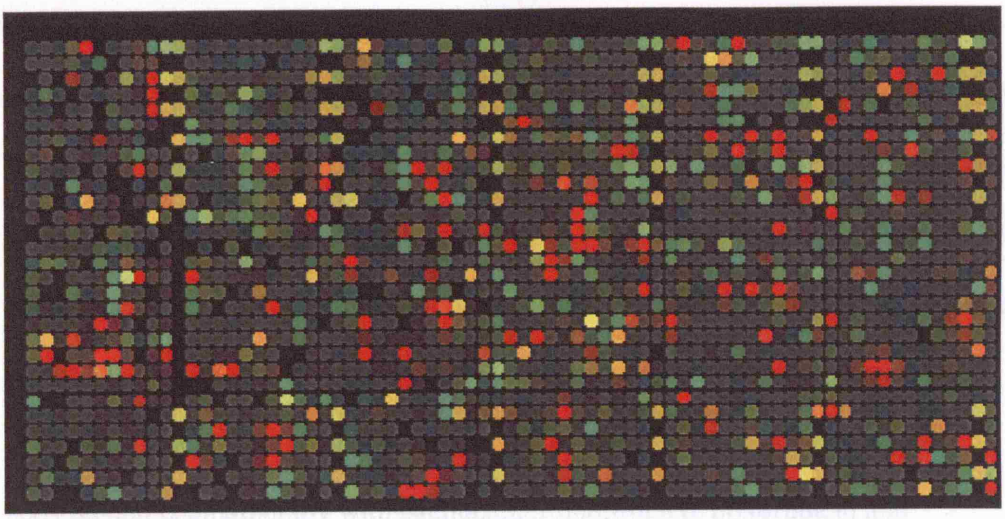


Figure 11 - Fluorescently labelled microarray image

1.4.1 Clinical applications

By profiling tumours, a molecular fingerprint of individual tumours types can be built up. This technique has already been used in a number of more common cancers [225, 226]. Any given profile could potentially be pathognomonic of that particular cancer so ensuring less room for diagnostic doubt [227].

Traditionally, tumours are categorised on the basis of histology. Even if a repertoire of antibodies are used to make an accurate histological diagnosis, the methodology remains crude: the staining pattern of cancer cells do not reflect the molecular complexicity underlying the different neoplastic processes, and tumours that look identical under the microscope, and even express the same markers, often have different responses to cytotoxic agents. We now know that specific mutations in c-kit or PDGFRά can predict responses to Imatinib, and specific mutations in EGFR1 can correlate with responses to Gefitinib or Erlotinib.

GEM with simple hierarchical clustering has already led to the identification of new classes of cancer that transcend the distinctions based on morphological appearance alone. Of note, GEM profiling and neural networks have been employed to

distinguish between the different "small round blue cell tumours" [228], identify subclasses of adenocarcinoma of the lung and breast with clinical implications [229, 230], provide prognostic useful information in the management of patient with acute myeloid leukaemia [231], identify genes which predict for survival in Follicular Lymphoma [232] and factors which predict for survival after chemotherapy treatment of Diffuse large B-cell lymphoma [233]. One study discovered a multigene model with 74 markers which predicted for complete pathological response following neoadjuvant chemotherapy with paclitaxel, 5 fluorouracil, doxorubicin and cyclophosphamide in breast cancer [234]. The therapeutic implication of this means cytotoxic treatment can be predicted beforehand sparing individuals from ineffective treatments whilst others can have their outcome optimised by using highly sensitive cytotoxic agents from the start. Another application is using gene expression profile data to predict for recurrence. One study used a multigene assay to predict recurrence after Tamoxifen treated node negative breast cancer [235] whilst another identified patients likely to relapse following surgery for Dukes B colon cancer where adjuvant chemotherapy is not used routinely. By identifying a 23 gene signature which predicted for recurrence those high risk patients could be selected for adjuvant chemotherapy to reduce their risk [236].

With increasing knowledge and application of this technology it is likely that specialised arrays will be offered that are specific for a tissue type (e.g., mammary gland chip), physiologic processes (e.g., apoptosis chip, angiogenesis chip, invasion chip), or a class of genes (e.g., suppressor gene chip, oncogene chip).

1.4.2 Limitations of the microarray technology

The current microarray technology utilises the RNA extracted from a lump of tumour tissue complete with its supporting stroma. There is increasing evidence however that this apparent normal tissue is more than an innocent bystander and in fact capturing pure cell populations from tumour tissue may not be necessary. Studies have shown that surrounding stromal tissue shares much of the gene expression profile of the tumour of interest [219-221, 237, 238] implying that the stromal cells have been recruited by the cancer cells. It may transpire it is no longer necessary to filter out the genes associated with surrounding stroma. In this thesis a comparison of pure human osteoblasts and osteosarcoma was initially made. As osteosarcoma arises from bone forming cells it was thought this was a good comparison. All but two of the samples were from post resection biopsies and not pure needle core biopsy samples. Some of the tumour contained blood vessels, supporting stromal cells and invading white cells. This prompted the use of a mixed reference probe containing human osteoblasts, endothelial, fibroblastic and lymphocytic cells for comparison. Culturing cell populations from fresh tissue was another approach to reduce contamination. However, cultured cells may not accurately represent the molecular events taking place in the actual tissue they were derived from. Assuming methods are successful to isolate and grow the tissue cells of interest, the gene expression pattern of the cultured cells are influenced by the culture environment and can be quite different from the genes expressed in the native tissue state. This is because the cultured cells are separated from the tissue elements that regulate gene expression, such as soluble factors, extracellular matrix molecules, and cell-cell communication. Thus, the problem of cellular heterogeneity has been a significant barrier to the molecular analysis of normal and diseased tissue. Attempts to obtain pure tumour cell samples have resulted in the genesis of several methods of cell enrichment, such as use of

primary cell culture as described above and microdissection of frozen or paraffin embedded tissue. Laser capture microdissection (LCM) has been developed to provide scientists with a fast and dependable method of capturing and preserving specific cells from tissue, under direct microscopic visualisation [239, 240]. With the ease of procuring a homogeneous population of cells from a complex tissue using the LCM, the approaches to molecular analysis of pathological processes are significantly enhanced. The mRNA from microdissected cancer lesions has been used as the starting material to produce cDNA libraries, microchip microarrays, differential display, and other techniques to find new genes or mutations. The development of LCM allows investigators to determine specific gene expression patterns from tissues of individual patients. Pure populations of cells can be obtained, RNA extracted, copied into cDNA, and hybridised to thousands of genes on a cDNA microchip microarray. In this manner, an individualised molecular profile can be obtained for each sample histologically identified [237]. Using such multiplex analyses, investigators will be able to correlate the pattern of expressed genes with the aetiology, premalignant state, pre-treatment and post treatment appearance. A patient's risk for disease and appropriate choice of treatment could, in the future, be personalised based on such a profile [235, 236]. Efficient coupling of LCM of serial tissue sections with multiplex molecular analysis techniques should lead to sensitive and quantitative methods to visualize three-dimensional interactions between morphologic elements of the tissue. For example, it will be possible to trace the gene expression pattern along the length of a prostate gland or breast duct in order to examine the progression of neoplastic development [237]. This will result in a new era in the integration of molecular biology with tissue morphogenesis and pathology.

1.4.3 Proteomics

Proteomics is the large scale, or high throughput analyses of proteins [241]. The biochemical display of large numbers of proteins on two-dimensional polyacrylamide gels dates back to the 1970's [242, 243]. However although the technique of two-dimensional gel electrophoresis was highly reproducible between laboratories, determining the identity of proteins was difficult because of the lack of sensitive and rapid analytical methods (such as PCR and the automated sequencer for DNA analysis) for protein characterisation. In the 1990's the emergence of biological mass spectrometry removed most of the limitations of protein analysis. Identification of most of the expressed human genes has been accomplished through the human genome project. This coupling of the powerful new analytical tools, plus the availability of the entire human coding sequence in public databases heralds a whole new era. Today the term proteomics also covers the functional analysis of gene products or "functional genomics" [242]. A cell is influenced by a wealth of metabolic and regulatory pathways for its survival and there is no strict linear relationship between gene expression and protein function. Deriving the molecular profile tells us about the gene products of a particular tissue or tumour. It does not tell us the functional state of that tissue or tumour. By deriving the protein repertoire of a tissue we gain information regarding the functional status of the cells or tissue. The detection of a particular gene expression profile does not imply that that gene is functional. Genomics can uncover clues to functionally important molecules and together with proteomics can generate information to tailor a treatment to the individual patient. It is important to take in account the dynamic state of the protein, such as the effects of post-translational modifications and protein-protein or protein-DNA interactions. Through an integrated genomic and proteomic analysis, the ultimate outcome will be a complete functional understanding of the molecular events

underlying normal development and disease pathophysiology. This higher level of functional understanding will be the basis for true rational therapeutic design.

1.4.4 Bioinformatics

Organising and making sense of all the data generated from microarray experiments has become a science of its own. Bioinformatics now has an essential role both in deciphering genomic, transcriptomic, metabolomic and proteomic data generated by high-throughput experimental technologies, and in organising information gathered from traditional biology [244, 245]. Several techniques have been used for the analysis of gene expression data, including hierarchical clustering, mutual information and self organising maps (SOMs). The computational tools necessary for analysing data are rapidly evolving and no clear consensus exists as to the "best" methods for revealing patterns of gene expression. It may be there is no one right approach for all experiments but a best fit for a particular experiment. The methods used in the analysis of the data generated in this thesis are described in chapter 2 and the results shown in chapter 3.

Chapter 2 Materials and Methods

2.1 Buffers, media and solutions

Deoxynucleotide triphosphate mix (dNTPs)	100mM deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP)
10x PCR Buffer (20mM magnesium chloride)	100mM Tri, pH 8.3, 500mM potassium chloride, 20mM magnesium chloride
Phophate-buffered saline (PBS)	137 mM sodium chloride, 2mM potassium chloride, 10mM sodium hydrogen phosphate (dibasic), pH 7.4
1% Agarose	1g RNAse free agarose, 100mls TAE, 3µl ethidium bromide
20x Saline sodium citrate (SSC)	3M sodium chloride, 0.3M sodium citrate, pH 7.0
Tris-acetate-EDTA (TAE)	40mM Tris pH 7.8, 20mM sodium acetate, 1mM ethylenediametetraacetic acid (EDTA)
Tris-EDTA (TE)	10mM Tris pH 7.4, 1mM EDTA
SDS	Sodium Dodecyl Sulphate
Alkaline phosphatase stain	4% New fuchsin, 2M hydrochloric acid (HCL) 4% sodium nitrite, buffer containing Tris HCL Naphthol AS-BI Phosphate. Mayer's Haematoxylin
0.5X TAE	50X TAE 100mls plus 9,900 mls millipore water
Staining buffer:	0.2M Tris10mls, 0.1M HCL 2mls, H_2O 28mls
Incubating medium	4% new fuchsin in 2M HCL 8 drops 4% sodium nitrite 8 drops Staining Buffer (as above) Naphthol AS-BI Phosphate 10mg
0.1% DEPC Water	500mls Millipore H_20water plus 50µl DEPC -autoclaved
TE pH 8.0	5mls 1M Tris HCL 1ml 0.5M EDTA 500mls millipore water-autoclaved
PCR megamix	DNA 2.0 µl 10X buffer 5.0 µl dNTP's (20mM) 0.5 µl + primer 0.1 µl - primer 0.1 µl Taq (DNA polymerase)0.5 µl H_2O 48 µl – total volume

2.2 Collection and storage of tissue

From December 1998 Dr Anne Sandison started collecting sarcoma tissue samples surplus to diagnostic requirements. These were stored in a tissue bank held in the Histology department in the Institue of Orthpaedics at the RNOH. All types of sarcoma tissue were collected not just OS. In October 1999 I was granted ethical approval to collect all high grade central osteosarcoma specimens surplus to diagnostic requirements from consenting patients. All tumour tissue was a gift donation from the patients once consent for use of surplus tissue had been obtained. A patient information sheet was provided which outlined the project and the reasons for wanting to use their tissue for this research. Specific information sheets were written for patients aged less than 16 years in addition to the information sheet provided for all consenting adults which was also suitable for parents/carers of minors (appendix 1). I established a pathway for the weekly identification of potential patients with OS who would undergo either a diagnostic biopsy, resection of primary tumour or metastastectomy. This involved contacting the junior member of the orthopaedic team every Monday afternoon following their ward round. On a Wednesday morning I would arrive outside the theatre in which the procedure was being undertaken and wait with my polystyrene collection box filled with ice. In theatres the surgeon would place the sample inside a sterile clear plastic bag and then double bag this with the histopathology request form and pass it to me outside theatres. I would place the sample in its bag on the ice and drive straight to RNOH 12 miles away. This would take me about 40 minutes. Samples arriving directly from the theatres or radiology at RNOH were transported immediately by the portering service to the histopathology department. On arrival at the department of histopathology all samples were checked in by the administrative staff. Dr Jean Pringle, Consultant Histopathologist was very supportive of my work and she would arrive promptly to meet me once the sample was

logged into the system. I would alert her in advance of my arrival. Dr Pringle taught me how to perform imprints on the clinically suspected OS samples I had couriered from UCLH. I performed all this work in the tissue cell culture room in a laminar air flow type two cabinet. This entailed the smearing of the fresh tissue sample to a sterile slide under aseptic conditions. The slide was air-dried and stained for alkaline phosphatase positivity. The laboratory staff performed the alkaline phophatase staining of all the imprint slides. Separate adjacent samples were collected for histological confirmation by Haematoxylin and Eosin (H&E) staining. This was performed by Dr Jean Pringle. A 0.5cm^2 portion of surplus tissue was placed under aseptic conditions into a cryofreeze tube. This was anonymised to an identification number written on the side of the tube. This tube was placed in a liquid nitrogen tank for storage and its location in the tank recorded in a separate folder. For the samples I collected I transported them back to the laboratory in a small tank containing liquid nitrogen. On arrival at the Wolfson Institute Biomedical Research (WIBR) I placed these samples in my designated tray in the laboratory liquid nitrogen storage tank. I kept the same histology identification number as their unique identifier. I collected four fresh tumour samples from four different patients between December 1999 and March 2000. In April 2000, an unexpected moratorium was placed on the collection of any new tissue for experimental use at the RNOH in response to the Alderhay Children's Hospital enquiry. Despite my project gaining prior ethical approval, it was a Trust policy to ensure procedures were in place to ensure all biological samples could be correctly accounted for. For the next nine months I worked through the samples previously stored by Dr Anne Sandison. This entailed working through the liquid nitrogen tank file identifying the location of the stored OS samples. I first made a list of the stored identification numbers then cross referenced these with their final histology report. Those that were confirmed high grade OS were compiled as a

separate list. I worked through the stored samples removing all samples on my confirmed list. I collected a total of 53 samples from 53 different patients. I transported these back to the WIBR and stored them in the laboratory liquid nitrogen tank. When the moratorium was lifted in January 2001 I was able to collect four more samples from four different patients. In total, 61 OS samples were collected. For the final 16 samples used in the microarray analysis I obtained their medical notes and collected all relevant clinical characteristics. This included age at presentation, gender, stage and site of tissue obtained. Information on the cytotoxic regimen and the timing of any chemotherapy received in their clinical course was also recorded.

2.3 Tissue Cell Culture

All reagents were supplied by GIBCO™ Invitrogen Corporation (Invitrogen, UK) with the following exceptions: 10% fetal calf serum (FCS, Helena Biosciences, UK), Dimethyl sulphur oxide (DMSO, Sigma, UK), Trypan blue 0.4%, collagenase type ll, 70% industrial methylated spirits (IMS).

2.3.1 Basic tissue cell culture

All procedures were performed in a laminar air flow type 2 cabinets. Tissue cell culture principles were learnt by using two osteosarcoma cell lines Saos 2 and MG63. Both osteosarcoma cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM GIBCO™ Invitrogen Corporation) supplemented with 10% fetal calf serum (FCS Helena Biosciences), 2mM L-glutamine (GIBCO™ Invitrogen Corporation), 200U/ml penicillin and 200 microgram/ml streptomycin (GIBCO™ Invitrogen Corporation) in T75 culture flasks (75cm^2; Corning). Primary human osteoblasts were supplied as a gift from Dr Lucy di Silvo. She isolated these cells from trabecular bone

establishing a protocol that is now used in the Institute of Orthopaedics RNOH Stanmore. Her methods formed the basis of her PhD thesis where she demonstrated Human Osteoblasts (HOBs) isolated from a human femoral condyle represented the osteoblast phenotype by demonstrating cell proliferation, alkaline phosphatase activity, the secretion of osteocalcin and the formation of mineralised nodules. The HOBs were grown in similar conditions using the same medium and supplementation under the same incubation conditions of 37°C with 5% Carbon dioxide. All tissue cultured cells were reviewed daily; the media changed every three to four days. When cells became 80% confluent they were washed, checked for viability using Trypan-blue staining and split using 1ml of Trypsin-EDTA (0.25%, 1mM EDTA.4Na) (1x). Cell viability using Trypan-blue staining was performed before cell counts to determine the level at which to re-seed future cultures. Cell kinetics of all cultured cell lines was performed to determine the mid exponential point of cell growth. Cells were harvested at the mid exponential point for RNA extraction. Cell counts were performed to ensure that consistent sized pellets were stored for later RNA extraction or in 90% FCS and 10% DMSO for later resuscitation. Human endothelial cells were cultured by Dr Hsei-Wei Wang who provided me with the cell pellet for RNA extraction. Dr Wang obtained his endothelial cells by tissue digestion of breast reduction mammoplasties. Positive selection of endothelial cells with magnetic beads coated with antibodies recognising endothelial specific markers (anti-PECAM antibodies, *Ulex Europeus agglutinin*) were performed. After isolation the cells required further characterisation by FACs (flow cytometry cell sorting and analysis) to confirm the expression of endothelial cell specific markers.

2.3.2 Primary cell culture

A 0.5cm² portion of confirmed osteosarcoma tissue was placed in a tube with 5mls 165U/ml collagenase type ll. This was then left overnight at 4°C. The following day the tube was placed in a water bath at 37°C. Every 5 minutes the mixture was agitated for a total of 50 minutes. After this time the tube containing the mixture was transferred to a laminar air flow type 2 cabinets and further agitation with a pipette undertaken. The mixture containing any undissolved fragments of tissue was passed through a sterile filter. The filtered mixture was centrifuged at 1600 rpm at 21°C for 5 minutes. The supernatant was removed and discarded. The cell pellet was resuspended with 5 mls of culture medium and placed in a T25cm² flask. 10 mls of supplemented culture medium was then added to the flask. The flask was left to incubate overnight at 37°C with 5% CO_2. The flask was checked daily for cell growth with regular changes of media every three days. When there was good cell growth with almost complete confluence of adherent cells, the cells underwent their first passage with some aliquots of cells re-seeded for further growth and other aliquots being placed in 6 well plates for confirmatory studies. After this first passage, any supernatant was collected before any further supplemented media changes. The 15 ml Falcon tube was labelled and stored at -80°C for further confirmatory studies.

2.3.3 Cryopreservation of cells

Following trypsinisation the cells suspended in the resulting solution was performed. The total volume of solution was divided into aliquots containing the desired number of cells for later resuscitation. The 15mls Falcon tubes containing this mixture were placed in the centrifuge at 4°C. These were centrifuged at 1600 rpm for 5mins. The supernatant was removed (stored if required) and the cell pellets washed in PBS. The tubes were again centrifuged following washing with PBS under the same conditions. Again the supernatant was removed and the cell pellet preserved. The cell pellet was

then resuspended in a mixture of DMSO and FCS, and placed in a small freezing tube for future use. The tube was first placed in a propanol containing freeze box before transfer to the liquid nitrogen tank the following day. This ensured the cells had time to acclimatise before storage in liquid nitrogen.

2.3.4 Resuscitation of cryopreserved cells

The supplemented DMEM medium was warmed to 37°C in a water bath. The vial of cells to be resuscitated was removed from liquid nitrogen whilst wearing protective clothing. The cap of the vial was loosened immediately to release any nitrogen trapped inside. This prevented the vial from exploding from any gas trapped inside and allowed to warm. The vial was incubated in the water bath at 37°C until the ice pellet was almost thawed. The vial was transferred to the laminar airflow cabinet for the remainder of the procedure. The vial was agitated gently until the pellet had thawed completely. A volume of warm medium equal to that of the thawed cell suspension was added to the vial and the volume of the vial was transferred to a 15 ml Falcon tube and incubated in suspension in the water bath for 5 minutes. Doubling volumes of warm media were added to the cells until the final volume reached 10 mls. Cells were incubated for 5 minutes after each doubling step. The cell suspension was centrifuged at 1600rpm for 5 minutes. The supernatant was removed, taking care not to disturb the cell pellet. Two mls of fresh supplemented media were added to the tube and the cell pellet agitated using a Pasteur pipette. A further 8 mls of media was added to the tube, mixed and recentrifuged. The supernatant was removed and discarded as before. 2mls of fresh medium was added to resuspend the cell pellet as before. A 5ml syringe and 23G needle were used to force the cell suspension through the needle several times to produce a single cell suspension. The volume was made up to 10 mls and the cells divided between the required numbers of flasks, depending on

the concentration of cells resuscitated. Supplemented medium was added as appropriate.

2.4 Alkaline phosphatase staining

Cells were seeded at a density of 1 x 10^5 onto coverslips, each one placed at the bottom of a six well plate. When the cells were confluent after three days the medium was removed and each well was washed with 5mls PBS. Incubating medium was made up from 4% new fuchsin in 2M hydrochloric acid (HCL), 4% sodium nitrite, buffer (see materials for make up) and Naphthol AS-BI Phosphate. This was incubated for 15 minutes at 37°C. The incubating medium was then discarded and the slides washed in distilled H_20 for 2 minutes. A counterstain (Mayer's Haematoxylin) was added and left to incubate at 22°C for 2 minutes. The slides were rinsed in distilled H_20 and mounted onto to microscope slides. A control sample of fresh not fixed liver tissue was run as a positive control. The endothelial cells within the liver tissue are high expressers of alkaline phosphatase activity.

2.5 Osteocalcin assay

The removed media after two to three days cell growth when cells were at 80% confluence was decanted into 15ml Falcon tubes. These were snap frozen and stored in the -80° freezer. I organised a courier to transport them in liquid nitrogen to Dr Brendan Jackson at the University of Sheffield who was an expert in determining serum osteocalcin levels using an immunoabsorbant assay. He used the supernatants of all five different primary cell cultures which I sent him. The final analysis of all samples is shown in (Table 3.2).

2.6 RNA extraction

2.6.1 Osteosarcoma Tissue

All equipment was autoclaved before use where possible or treated with diethyl pyrocarbonate (DEPC) H_20, to ensure everything was RNase-free. DEPC is a strong inhibitor of RNases. For fresh frozen tissue RNA extraction, the vial containing the frozen piece of tumour tissue was removed from the liquid nitrogen storage and placed on ice. The lid was loosened immediately and the specimen transferred to the category 2 laminar air flow cabinet. The tumour was removed using a sterile needle and placed in a 50 ml Falcon tube containing RLT buffer (GITC Guanidine isothiocyanate containing) supplied by RNeasy. For each 30mg of tissue, 600µl of buffer was required. ß-mercaptoethanol (ß-ME) was added to the RLT buffer before use as per manufacturer's instructions. The specimen was initially homogenised using a rotary star homogeniser for two minutes then underwent further homogenisation using a Q1A shredder (Qiagen® Ltd, West Sussex UK). RNA extraction followed as per manufacturer's protocol using an RNeasy mini kit (Qiagen® Ltd, West Sussex). The principle of this technique is that the tissue is disrupted by a highly denaturing solution and homogenised. Ethanol is added which promotes selective binding of RNA to the RNeasy membrane. The contaminants undergo further washes with supplied buffers and high quality RNA is eluted in H_20.

2.6.2 Cultured cells

For all cell lines used, including Saos 2, MG63 and the cells lines which made up the reference probe, in addition to primary cell cultures of osteosarcoma tissue, the RNeasy Mini kit was used as per manufacturer's instructions. This followed in principle the same protocol for extraction of RNA from tissue, but the rotary star homogeniser was not required for full disruption of the tissue.

When cells were ready for RNA extraction the old medium was removed. The cells were washed with 10mls Phosphate Buffered Saline (PBS) (GIBCO™ Invitrogen Corporation). 5mls of trypsin 0.25% was added and the flask returned to the incubator for 5 minutes. The flask was checked for loss of adherence of the cells. When all cells are in solution 5 mls of medium was added to inactivate the trypsin. The mixed solution of suspended cells was transferred to a 15mls Falcon tube. This was centrifuged at 1600rpm and 4°C for 5 minutes. The supernatant was removed and the cell pellet washed with 5mls of PBS. This was centrifuged again under the same conditions. The supernatant was removed and the cell pellet mixed with 2mls of PBS and transferred to a small cryotube. This cryotube containing the cell pellet underwent a final ultracentrifugation for 2 minutes before the PBS was removed. The tube was coded and immediately placed in liquid nitrogen if RNA was to be extracted at a later date. If RNA was to be extracted immediately the cell pellet was lysed with 600µl of guanidine thiocyanate containing buffer as supplied by RNeasy Mini kit™.

2.7 Agarose gel electrophoresis

Integrity of total RNA was confirmed by the presence of 28S and 18S ribosomal bands on 1% agarose gels. 1% agarose gels were made up using 1g of RNAse free agarose added to 100mls of TAE containing 3µl of ethidium bromide. Each lane contained 1µg of RNA loaded with 1µg of orange running buffer. They were run for 20 – 30 mins before images were checked with UV light. Two bands representing the 28S and 18S ribosomal bands were identified (Figure 3.2).

2.8 RNA quantification

Quantification and purity of RNA was assessed by spectrophotometry. 2µl of RNA was added to 500µl of distilled H_20 before the mixture was transferred to a glass

cuvette for analysis. Absorbance was read at both 260nm and 280nm. A ratio 1.7 or greater was deemed suitable for cDNA probe generation (Table 3.2).

2.9 cDNA probes

A general reference probe thought to be representative of most cell types present in the bone/tumour/stromal environment was made up as follows: 1 x 10^6 cells each from human osteoblasts, human endothelial cells and peripheral mononuclear cells were used to obtain RNA from each type of cell. The peripheral blood mononuclear cells were a donation from Dimitra Bourboulia a PhD student working with me in the laboratory. Briefly she collected blood in a tube containing EDTA from healthy volunteers. To separate the peripheral blood mononuclear cells (PBMCs) she diluted the re-constituted blood with the same volume of buffer A. She added to one 50ml Falcon tube 1 part of Histopaque and 2 parts of the diluted blood. She layered the diluted blood on to Histopaque centrifuged at 1,800rpm for 30min with no brake and no acceleration at room temperature. She removed the PBMC band (middle layer) in to one 15ml or 50ml falcon, and topped up to 15ml or 30 ml with buffer A. She pelleted the cells at 1,500rpm for 10min, room temperature with acceleration and brake. She carefully took off supernatant (SNT).

The RNA was extracted as per manufacturer's protocol using an RNeasy mini kit (Qiagen® Ltd, West Sussex UK). 5µg of total RNA from each of the cell cultures was mixed making a total volume of 15µg. 5 µg of this mixture was used to make each cDNA probe. The same reference sample was used to compare against each of the individual tumours, thus creating an unchanged reference value for each individual gene.

Single stranded cDNA was made as per Research Genetics protocol, but essentially I used Superscript ll (Life Technologies) to generate the cDNA from Oligo dT (1μg/μl 10-20 per mixture, Research Genetics, Huntsville, AL) primed total RNA. 5μg of total RNA was used to make each single probe. Two replicate probes were made from one pool of RNA from each individual patient. The probe was radiolabelled with ^{33}PdCTP (ICN Radiochemicals) then purified from unincorporated nucleotides using a Biospin 6 column (Bio-Rad. #732-6002). Counts of the labelled probe were checked to determine uniformity across all samples.

2.10 Microarray hybridisation

The radiolabelled probe was hybridised overnight to a pre-hybridised nylon filter. Pre-hybridisation in a solution of MicroHyb (Research Genetics, Huntsville, AL) to which Human Cot-1 (Human Cot-1 DNA, Life technologies) and poly dA (Research Genetics Cat. #POLYA.GF, 1μg/μl, Huntsville, AL) were added was performed for at least 2 hours. This step decreases the incorporation of repeat sequences thereby reducing the background of the resultant filters. Following 18 hrs hybridisation with the radiolabelled probe the filters were washed using 0.5-1% concentrations of Sodium Dodecyl Sulphate (SDS) and 2X-0.5X SSC to remove any non-hybridised probe.

The hybridised filters were placed on blotting paper moistened with distilled H_20. They were aligned, so as not to overlap, and wrapped in cling film. The filters were placed in a cassette and the phosphor screen aligned on top. The phosphor screen was held securely with the use of sticky tape externally. The cassette was closed and left untouched for 48 hours. This was found to be the optimum time for probes made with 5μg total RNA by previously exposing filters from 24 – 72 hours. After this time the phosphor screen was scanned using a Cyclone™ (Packard Instrument Company,

Meridan, USA) imager and the image generated imported into Pathways™ software for analysis.

2.11 Stripping and storing of Genefilters™

500mls of 0.5% SDS was poured into a plastic container. The solution was heated in a microwave oven for 7-10 minutes until boiling. The Gene filter membranes were placed into this solution, the container covered and briskly agitated for 1 hour. Following stripping, the filters are placed on blotting paper moistened with T.E. (pH 7.4). The stripping efficiency was checked with a Geiger counter. The filters could be re-used up to five times. The filters left on blotting paper moistened with T.E. were stored at 4°C.

2.12 Scanning and data extraction

The phosphor screens were scanned at 635nm with 50μm resolution using a Storm 860 phosphor imager (Amersham Pharmacia Biotech, UK). The PMT voltage (sensitivity) was set to 1000v. The signals from the array probes were quantified using Pathways™ software. The software created a template by using control spots to identify correct alignment of the filter. There is a tool bar which allows enhancement of the image so the control points can be seen more clearly. This does not affect the stored data; it is merely a tool to allow more accurate alignment of the filters. The alignment wizard is useful to locate the four main control points in a clockwise fashion. The software then calculates the grid created to make sure the alignment is within the tolerance range. Each of the inside angles of the bounding rectangle must be 90° (+/-1°) and the overall skew no greater than +/-10°. If the image does not fit the criteria it is automatically rejected by the software programme. Once the alignment is accepted or corrected the position of each of the 16 control alignment

control points was fined tuned (Figure 3.4). Once the image is imported, information regarding the nature of each specific filter is entered. The gene filters used in this research study were GF211™. It is imperative that this is correctly entered during the importing process, as different filters have different data sets. The data were exported to Excel (Microsoft, UK).

2.13 Analysing an image

In the Pathways software one can perform either single filter or comparative filter analysis. For the purposes of this thesis comparative datasets were stored with each sample filter compared to the same reference filter (new ref C14). This allows normalisation of all values so filters can be linked and relationships between the filters determined. The software allows the researcher to choose to normalise using all data points or only the control positives. Normalisation involves calculating the average of the "control points or data points", and using that average to calculate a normalisation factor with a simple mathematical equation. Then the raw intensity for each spot on the image is multiplied by that normalisation factor to obtain normalisation values. It is the normalised values which are recommended by Pathways™software to be used in all calculations when comparing filters. There are no advantages to using "all control positives" or "all data points" for normalisation. Either is acceptable but if any of the filters have particularly bright spots, which may skew the data, then normalising using "all points" is recommended. The software allows visual comparison of all the data by pseudocolours either as a red/green overlay or red/blue overlay. The red/green uses one image as the red channel and the other as the green channel. It then blends both images so there is one final colour. This is based on the ratio of the intensities of both images.

All Red/minimal green = red

Minimal red/all green = green

All red/all green = yellow

Minimal red/minimal green = black

The red/blue overlay subtracts one image from the other. Positive values are shaded red while negative ones are shaded blue.

All Red-No Blue = Red

No Red- All Blue = Blue

No Red – No Blue = White

All Red- All Blue = White

The toolbar allows customisation of the image such as removing housekeeping genes, control points or choosing certain genes in the thumbnail inspection frame. Using the histogram intensity distribution toolbar one can view the distribution of all the values on both filters being compared (Figure 3.5). If these follow a normal distribution (Figure 3.6) then it was deemed the data points were normally distributed so they were accepted into the data set for comparison. If they were skewed, they were rejected and no text file was created for importing (Figure 3.7).

2.14 Creating Tab delimited text files of data

Report files are generated by the Pathways™software. By using the synfilter button on the toolbar, particular data points, housekeeping genes, control points are excluded from a report. Using the report wizard tabulated delimited text files are generated and exported to a file of choice. The data files are opened in Microsoft excel, before focussing on variables of interest. Essential information such as GenBank Accession

number, name of gene and normalised value for each gene, is saved as a new text file labelled appropriately for that particular sample. Text files were created for 22 tissue samples and the two cell lines. 16 of the 22 tissue samples had data for both replicates. These files were used for statistical analysis in Microsoft excel calculating the mean, median, skew and kurtosis (peakiness). Correlation co-efficients for each of the replicates were derived. If both filters had correlations co-efficient of 0.8 or greater they were accepted for further analysis. A combined value for each gene was created by averaging the value for each gene in the replicates and making this the final value for that sample. Following further statistical analysis of the replicates and filters a final data set of 16 tissue samples and two OS cell lines were achieved.

2.15 Preparing data for hierarchical clustering

The tabulated delimited text files of the normalised data for each of the samples were imported into Microsoft access. Using the query function with the gene title to link all values for the respective samples, large spreadsheets of all the normalised values for each individual gene could be generated. The spreadsheet comprised 19 columns for 4324 genes. The 191 housekeeping genes were deleted leaving 4133 genes for 19 samples including the reference sample. In excel, ratios were created for each gene by dividing each sample's value by the reference for that particular gene. This created a spreadsheet with ratios of 4133 genes for 18 samples.

Cluster™ and Treeview® are two software programs written by Michael Eisen and downloaded from the Stanford University website (http://www.smi.stanford.edu/)[246]. They were written to provide computational and graphical tools to allow analysis of data from microarray experiments. The programme Cluster™ organises the data in a number of ways for e.g. hierarchical

cluster dendrogram or self organising maps (SOMs). Data are loaded as ratios in the form of tabulated delimited text files with the first column being the gene title or identifier and subsequent columns being the additional samples. The rows represent the genes. Data can be adjusted in Cluster™ if required. Average linkage hierarchical clustering of the $\log_2$ transformed ratio values was performed using Cluster™ after filtering the genes to remove non-varying data (SD<0.5 of log transformed values). The aim of this was to assemble the genes ratio values from each OS sample into a tree. No further normalisation of genes or arrays were performed as no time course experiment had taken place. Samples joined by short branches are similar (gene expression profiles similar) to each other, whereas increased length branches indicate samples are more distantly related. Three output files are generated .cdt (clustered data table) .gtr (gene tree) and .atr (array tree).

Treeview® is the program that allows interactive graphical analysis of the results from cluster. It loads the .cdt file and graphically represents the data as a dendrogram with red for up-regulated genes, green for down regulated genes and black for those genes with a similar expression ratio as the reference probe. The relationship of the samples is viewed horizontally at the top of the dendrogram and the genes are shown vertically (Figure 3.9).

It is possible to highlight areas of significance and interest from the pictorial representation and store the gene names as text files and the images as bitmaps.

2.16 Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)

PCR consists of a chain reaction mediated by DNA dependent DNA polymerase, which results in the exponential increase of copies of a target DNA sequence. I used Taq polymerase a thermostable DNA polymerase which can resist temperatures up to 96° C without becoming denatured. This means Taq polymerase can withstand the thermal cycling necessary to continuously denature and re-anneal the strands of synthesised DNA. cDNA was made as in Materials and methods section 2.9 for generation of cDNA probes with the following exceptions. The RNA underwent DNA clean up first as outlined in section 2.19. This is an important additional step to ensure that the only DNA present in the PCR reaction is the desired target sequence. The protocol and commercial RNA extraction set I used did have the property of ensuring nuclei were removed so no genomic DNA should be present anyway. All PCR experiments took place in a specified clean "PCR "room used only for this purpose. All reagents except target DNA were added in there. UV irradiation of all reaction tubes and H_2O was undertaken prior to any reaction. Cold non– radiolabelled dCTP was used along with the other nucleotides. Mega mixes for the separate addition of ezrin and actin primers were made up as in section 2.1. (Buffers, media and solutions). The samples were placed in a PCR machine (MWG-Biotech Primus 96 Thermal Cycler). For actin the annealing temperature T_m was 52°C, and for the ezrin primers it was 56°C. After the lid was closed, the lid heat was turned on to prevent condensation. The programme ran at 96°C for 2 minutes, and then entered the amplification cycle: this was 95°C for 30 seconds, T_m for 45 seconds and 72°C for 60 secs. The samples each underwent a total of thirty cycles before running at 72°C for 10 min. The lid heat was then turned off and the samples held at 4°C until entry into the fridge at 4°C. The products generated were run on a 1% agarose gel containing 0.2μg/ml ethidium bromide using a 1KB DNA ladder to identify a product of 349bp

for ezrin and 400bp for actin (Figure 3.10). The products were purified and sequenced to confirm their identity.

2.17 Ezrin Primers

The ezrin primer was designed by eye according to a set of generally accepted rules for good primer design. Natalie Wilder a PhD student in the laboratory guided me through the design based on her method and experience. The primer had to be at least 16 nucleotides long, to ensure uniqueness within the template DNA. The G+C content of the primer should be as close to 50% as possible. The primer should end with a G or C nucleotide, to ensure strong annealing of the primer to the template for the initiation of extension by Taq polymerase. Runs of more than 3 of the same nucleotide should be avoided as well as sequences that could form secondary structures such as hairpins. A pair of primers used together for the amplification of a particular target sequence should be designed to have the same annealing temperature (T_a) of 50-60°C. This is calculated by the following formula: T_a (°C) =4(G+C) + 2(A+T). I designed the ezrin primers using the whole ezrin sequence. Ezrin primers used were; 5'- GCGGCATGGAATCCACC-3'; 5'- CCCTCTAAACCATGGCAC – 3'.

The primer sequence was checked in a NCBI blast search (www.ncbi.nih.nlm.gov) for high specificity before ordering.

2.19 Actin primers

These were donated by Natalie Wilder. The purified product was sequenced and confirmed to be actin.

2.19 RNA clean up

Each of the samples to undergo DNase were separately placed in eppendorfs and labelled. A proportionate volume of buffer and DNase were added to each volume of RNA. DEPC water was used to bring each volume up to 100µl. To each sample 100 µl phenol was added and vortexed. The tubes were each centrifuged for 5 minutes at 13000 rpm. The upper phase was removed and transferred to another eppendorf for the remaining steps in the procedure. 100 µl of chloroform was added and this mixture vortexed. Again the sample was centrifuged at 13000 rpm for 5 minutes. The upper phase was removed and to it added $1/10^{th}$ ammonium acetate and 2.5 volumes of 100% ethanol. This mixture was vortexed and stored at -80°C for 30 minutes. A further centrifugation step at 4°C at 13000 rpm for 5 minutes was done. Any RNA which had been cleaned up was seen as a small white pellet in the bottom of the tube. The supernatant was removed and the pellet washed with 100% ethanol.

2.20 Immunohistochemistry

Slides were prepared by Dr Jean Pringle on 7 tumour samples corresponding to the data set. The conditions were optimised to pH 9.9 with the ezrin antibody at a concentration of 1:500. I understand the preparation for this work entailed cutting 3µm thick sections from the paraffin-embedded samples, dewaxed in xylene, rehydrated and then pressure cooked for 2 minutes in antigen-unmasking solution (Vector Laboratories, Peterborough, UK). The iVIEW™ DAB Detection kit and NexES immunohistochemistry system (Ventana Medical Systems, Inc. Tuscon, USA) was used according to the manufacturers instructions. Briefly, the endogenous peroxidase activity was inhibited and the sections incubated with the ezrin antibody for 30 minutes, followed by a biotinylated Ig secondary antibody (Ventana Medical Systems, Inc. Tuscon, USA) and a streptavidin – horseradish peroxidase conjugate

(Ventana Medical Systems, Inc. Tuscon, USA). Finally a copper DAB (Ventana Medical Systems, Inc. Tuscon, USA) was applied. Sections were counterstained with Harris@ Haematoxylin (HD supplies, UK), dehydrated and mounted with a glass cover slip. This work was performed by Damien Hewitt (scientific officer) and the images generated of the immunohistochemical staining were taken by him also. The results are seen in Chapter 3 (Figures 3.11-3.15).

2.21 PCR product purification and DNA sequencing

This procedure was performed for both the ezrin and actin products using the QIAquick Gel Extraction Kit (QiagenLtd) as per manufacturer's instructions. In essence the DNA band was cut from the agarose gel with a clean sharp scalpel and dissolved in a buffer. The solution was passed through a column designed to bind DNA. Washes are performed to remove any remaining agarose after which the DNA is eluted using a supplied elution buffer. Aliquots of the purified product were sent for sequencing by Dr Ross Dunbar and team. The sequence derived was entered into the NCBI blast search.

Chapter 3 Results

This results chapter is presented chronologically starting with the outcome of the collected tissue samples. I will then describe the outcome of each of the experimental steps I undertook to order to obtain the final microarray data.

3.1 Clinical material

Table 2 shows all 61 samples used in this research work. Because of the unexpected moratorium placed between April 2000 and January 2001 on the collection of new fresh tissue samples from the RNOH, I used OS samples from the tissue bank collected by Dr Ann Sandison in addition to the samples I collected myself. I had a total of 53 archive samples and 8 fresh samples. I performed a cross checking identification search, entering the stored histological number from the sample with the final histology report. 60 of the 61 samples were extremity OS. One sample was a primary OS of the jaw and was not used in further analysis. I was able to extract RNA from 30 of the 61 samples. All 31 samples that did not yield RNA were all archived samples. I performed the RNA extraction using the same method for all samples. I was able to extract RNA from all freshly collected samples. As I was responsible for their collection, delivery to the pathology department and subsequent storage it was my conclusion that previously stored samples did not undergo the rigors of this approach. 25 RNA samples were of sufficient quality to generate cDNA probes. 22 cDNA probes were of good enough quality to apply to the microarray. Table2 summarises the origin and success of RNA extraction, cDNA probe generation and eventual microarray hybridisation. The clinical characteristics of the final 16 patients whose expression values contribute to the hierarchical dendrogram are shown in Table 3.

Sample number	Sample Id	Fresh or archive sample?	Was RNA extracted?	Was a cDNA probe made?	Was a microarray made from cDNA probe?	Was microarray data used in final analysis?
1	60738	f	y	y	y	Y
2	60040	f	y	y	y	Y
3	60181	f	y	y	y	Y
4	63139	f	y	y	y	Y
5	63125	f	y	y	y	Y
6	63111	f	y	y	y	Y
7	61124	a	y	y	y	Y
8	60147	a	y	y	y	Y
9	60131	f	y	y	y	Y
10	59576	a	y	y	y	Y
11	58720	a	y	y	y	Y
12	57848	a	y	y	y	Y
13	56307	a	y	y	y	Y
14	54876	a	y	y	y	Y
15	54283	a	y	y	y	Y
16	54397	a	y	y	y	Y
17	59124	a	y	y	y	N
18	63290	a	y	y	y	N
19	63264	a	y	y	y	N
20	59605	a	y	y	y	N
21	58610	a	y	y	y	N
22	54850	a	y	y	y	N
23	63230	a	y	y	n	N
24	59427	a	y	y	n	N
25	56265	a	y	y	n	N
26	56978	a	y	n	n	N
27	oromax	f	y	n	n	N
28	63385	a	y	n	n	N
29	60450	a	y	n	n	N
30	59442	a	y	n	n	N
31	63438	a	n	n	n	N
32	60739	a	n	n	n	N
33	60411	a	n	n	n	N
34	60396	a	n	n	n	N
35	60370	a	n	n	n	N
36	60177	a	n	n	n	N
37	60119	a	n	n	n	N
38	60110	a	n	n	n	N
39	60105	a	n	n	n	N
40	60052	a	n	n	n	N
41	59883	a	n	n	n	N
42	59718	a	n	n	n	N
43	59539	a	n	n	n	N
44	59421	a	n	n	n	N
45	59420	a	n	n	n	N
46	59064	a	n	n	n	N
47	58675	a	n	n	n	N
48	58637	a	n	n	n	N
49	57883	a	n	n	n	N
50	57238	a	n	n	n	N
51	57083	a	n	n	n	N
52	56577	a	n	n	n	N
53	56514	a	n	n	n	N
54	56110	a	n	n	n	N
55	56065	a	n	n	n	N
56	54874	a	n	n	n	N
57	54720	a	n	n	n	N
58	54691	a	n	n	n	N
59	54404	a	n	n	n	N
60	54256	a	n	n	n	N
61	41847	a	n	n	n	N

Table 2 - RNA sample origin

My original aim was to collect paired pre and post -chemotherapy samples on each patient. In addition to the problems brought about by the moratorium as described above on two occasions I collected pre-chemotherapy needle core biopsy samples which then did not have enough tissue surplus to diagnostic purposes to enable me to keep a sample for analysis. I therefore ended up using as many samples from which I could obtain good quality RNA for the microarray analysis. As you can see from the results in Table 3, I had 7 post chemotherapy samples which each had undergone less than 90% necrosis, two diagnostic needle core biopsy samples, three primary resection samples and five samples following metastatectomy. One lady was aged 71 years but the remainder of the cohort was aged between 7 and 23 years. There were 8 female samples and 8 male samples. Osteoblastic OS was the most frequent histological subtype followed by common OS with one sample each from fibroblastic, mixed differentiation and telangiectatic. Only four patients were chemotherapy naive. Sample met-c738 was the primary cell culture sample derived from the tissue rib metastases met-738. Three patients, met-738, post-139 and met-848 were all survivors of childhood acute lymphoblastic Leukaemia. I discovered this fact going through their medical notes to collect clinical details. Unfortunately there was no detailed information about this previous diagnosis, relevant family history or any information relating to previous treatment. My project allowed me access to the notes of the relevant patients from whom I had successfully extracted RNA. It did not allow me to approach any of the patients or their families retrospectively to collect further data. I think this would have been quite unethical and harmful for me to do so.

Id Number	Age	Gender	Histological Subtype	Type of sample	Time elapsed since last cytotoxic treatment	Cluster
Loc-131	7	F	Osteoblastic	Diagnostic needle core biopsy	4 months	B
Pre-147	12	F	Mixed differentiation	Diagnostic needle core biopsy	None	C
Pre-283	14	M	Common	Primary resection sample	None	C
Pre-124	71	F	Osteoblastic	Primary resection sample	None	A
Loc-181	14	F	Common	Primary resection sample	None	B
Post-720	8	M	Common	Post chemotherapy resection <90% necrosis	2-3 weeks	A
Post-125	16	F	Fibroblastic	Post chemotherapy resection <90% necrosis	2-3 weeks	B
Post-876	22	M	Common	Post chemotherapy resection 70% necrosis	2-3 weeks	B
*Post-139	16	F	Osteoblastic	Post chemotherapy resection <90% necrosis	2-3 weeks	B
Post-397	18	M	Osteoblastic	Post chemotherapy resection <90% necrosis	2-3 weeks	B
Post-307	23	M	Common	Post chemotherapy resection 50% necrosis	2-3 weeks	B
Post-111	13	F	Osteoblastic	Post chemotherapy resection <90% necrosis	2-3 weeks	B
*Met-848	14	M	Telangiectatic	metastatectomy	2-3 weeks	B
Met-040	12	F	Osteoblastic	metastatectomy	2-3 weeks	B
Met-576	16	F	Common	metastatectomy	14 months	A
*Met-c738	13	M	Osteoblastic	metastatectomy	23 months	A
*Met-738	13	M	Osteoblastic	metastatectomy	23 months	A

Table 3 - Summary of patient characteristics

* Survivor of childhood Leukaemia

3.2 Primary Cell Culture

When I began this project the plan was to extract RNA from several fresh OS tissue samples and compare their collective gene expression profiles. The system I used of nylon based arrays meant I needed a reference sample with which to compare each of the patient sample. The major publications at that time which had successfully completed this task included Chuck Perou's molecular portrait of breast cancer [247]. In his paper he created a standard reference sample by mixing 11 commercial cell lines from a variety of human cancers. He then used this mixed RNA pool to create the reference cDNA probe. I contacted Dr Perou by e-mail to explain my project and ask his advice about how I should create a reference probe for my OS samples. His advice was that if I could be confident that the OS samples I used consisted purely of OS cells then it would be reasonable to compare each of these tumours to a cDNA probe made from pure human osteoblasts as OS is an OB derived cancer. If I felt the OS samples, like breast cancer samples were more than likely to be contaminated by host cells then I should create a reference probe containing the cells most likely to be contained in a human OS sample. The most likely other cells present in an OS sample in addition to OS cells I felt would be leucocytes and endothelial cells. As OS is derived from the OB lineage I felt the most pure reference sample should contain a mixture of OB, endothelial cells and leucocytes. Dr Lucy di Silvio donated her well characterised Human Osteoblast-like cells (HOBs). To this I added the endothelial cells donated by Dr Wang and the PBMC donated by Dr Bourboulia. However what if I could culture primary cells from the fresh OS samples I had collected? If I was successful in this task I could then compare the gene expression profile of the corresponding pure primary cell culture of a tumour and directly compare it to the gene expression profile obtained when I made a probe

from the human tissue containing all the host cell contaminants. This could then allow me to subtract the host cell populations from the expression profile of the tumour and the theory was I could be left with a signature representing OS only. I used the primary cell culture technique as described in the method section on four fresh samples, oromax, met-738, met-843 and met-040. The primary cell culture obtained from met-738 was the most robust able to undergo cryopreservation and resuscitation without going into cell crisis. I was able to perform electron microscopy, alkaline phosphatase staining and supernatant measurement of osteocalcin on this sample. In Figure 12 I show the results I obtained from the primary cell culture from an os rib metastasis. The positive findings from each of these studies support the theory these cultured cells have been derived from an osteoblastic cell lineage.

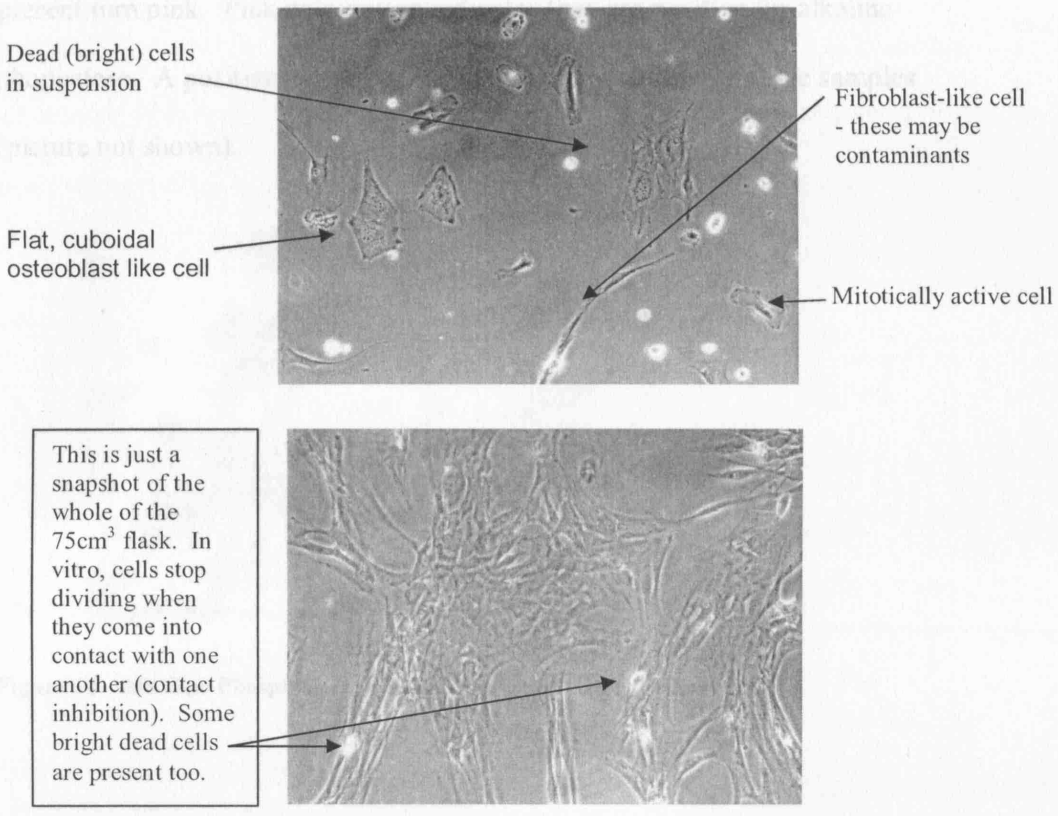


Figure 12 - Primary osteosarcoma cells

Primary osteosarcoma cells cultured from tissue met-738 (met-c738). The top image shows the appearance of the cells after 24 hours. They are beginning to adhere and divide. After five days their growth is almost confluent (bottom image).

3.3 Alkaline Phophatase staining

The primary cell culture met-c738 was seeded at a density of 1 x 10^5 onto cover slips and each placed at the bottom of a 6 well plate. The growth of the cells was checked daily and after three days growth I brought the 6 well plates to the Institute of Orthopaedics at RNOH to perform alkaline phophatase staining. I performed the procedure according to the protocol devised by Caroline Clifford as described in the methods section 2.4. The following picture demonstrates all cells present turn pink. Pink colouration indicates they are positive for alkaline phophatase. A positive control of cardiac tissue was run with these samples (picture not shown).

Figure 13 - Alkaline Phosphatase staining of primary OS cultured cells

3.4 Osteocalcin assay

The supernatants of the following samples were collected and frozen as described in the Methods section 2.5. The following table demonstrates the level of osteocalcin measured on two separate samples from each of the different tissues cultured. The normal range is 0-10 ng/ml. The HOB cells are the primary human osteoblasts donated by Dr Lucy di Silvio. She fully characterised these cells for her PhD as they record a level of 8.53 and 9.41 respectively. Osteocalcin or BGP (bone Gla protein) is exclusively found in bone tissue, accounting for 10-20% of the non-collagenous protein in bone. While the *in vivo* function of osteocalcin is unknown, its affinity for bone mineral constituents implies a role in bone formation. Hence it has been shown that osteocalcin is a biochemical indicator of bone turnover. The other primary cell cultures including met-c738 all produce high levels of osteocalcin so demonstrating the cells I have cultured are all capable of forming bone. This result confirms the cells I have cultured must predominantly be the bone forming OS cells from each sample and not contaminating stromal cells.

Sample id	**1st assay (ng/ml)**	**2nd assay (ng/ml)**
Oromax	12.54	11.58
Met-c843	1.28	1.60
Met-c040	19.36	18.71
Met-c738	18.71	20.42
Primary human osteoblasts (HOBs)	8.53	9.41

Table 4 - Osteocalcin assay results

3.5 RNA extraction

3.5.1 RNA integrity

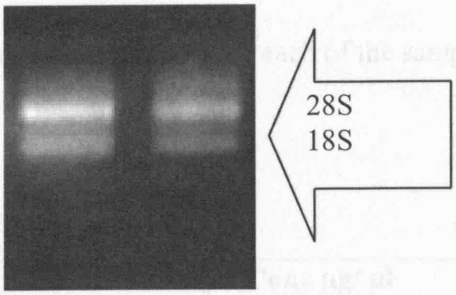


Figure 14 - Ribosomal bands

1% agarose gel showing clear ribosomal bands at 28S and 18S. The 28S band should be present in twice the amount of the 18S band.

RNA extraction was performed on 61 tumour samples. A 2µl representative sample from each of the 61 samples was run on a 1% agarose gel with 2µl buffer. 30 samples had good ribosomal bands as demonstrated above in Fig 14. No ribosomal bands were seen in the remaining 32 samples. Only RNA which demonstrated ribosomal bands 28S and 18S was kept for further analysis.

3.5.2 RNA purity

The ratio between the readings taken at 260nm and 280nm (A_{260}/A_{280}) provides an estimate of the purity of RNA. The A_{260}/A_{280} ratio is influenced by the pH. All the following estimates were made using 10mM Tris-Cl, pH 7.5. I calculated the concentration of RNA present in each sample by using a dilution factor of 2 in 500µl. Plus it is known that when a A_{260} has a value of 1 this equals a concentration of 40µg. I performed this calculation on all samples. Some RNA samples

yielded much more RNA than others. To make a successful cDNA probe needed at least 5µg of RNA per sample. Those samples with a low concentration such as pre-147 I only had enough RNA to make one cDNA probe. Unlike some of the samples where I could make replicates for each of the samples so increasing the accuracy of my data.

Id Number	Conc µg/ µl	260/280nmratio
Loc-131	1.43	1.88
Pre-147	0.15	1.875
Pre-283	0.32	1.64
Pre-124	0.61	1.80
Loc-181	0.40	2.35
Post-720	0.68	2.00
Post-125	2.10	1.62
Post-876	0.63	1.85
*Post-139	0.37	2.3
Post-397	0.30	1.76
Post-307	0.19	Not recorded
Post-111	0.50	Not recorded
*met-848	0.96	1.745
Met-040	1.09	1.92
Met-576	0.8	2.2
*met-c738	1.04	2.00
*met-738	0.75	1.72

Table 5 - Concentration and purity of the RNA samples

3.6 DNA probes

cDNA probes were generated from 25 of the 30 RNA samples. All measured at least 20-50 cps with a Geiger counter. Detailed counts were performed on met-c738 and met-738 which showed that a reading of 50 cps with a Geiger counter equalled 50 000 counts per minute on the Wallac 1409 Liquid Scintillation Counter (Perkin Elmer Life Sciences, Cambridge UK). The entire purified radiolabelled probe was used per sample with no equilibration of probe between samples.

3.7 Microarrays

3.7.1 Analysing and importing an image

The pathways ™ program was capable of reading these specially encoded TIFF images. The software used the position of 16 control points to align each of my images onto a clone grid. The thumbnail below demonstrates how I fine-tuned the position of each grid by centering the crosshairs on the control point. I needed to verify all sixteen control points.

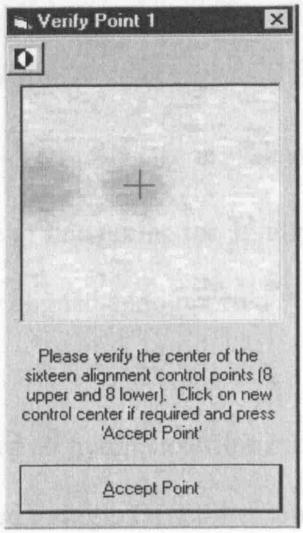


Figure 15 - Thumbnail verification point

When this was complete the Filter Import wizard in the pathways ™ program opened to collect specific information such as the filter type I had used, the name of the tumour sample, a description of its origin and finally my name as the researcher. The image below shows the analysis grid confirmation. This is an auto-centered grid that has estimated the spot centres as I aligned them. I needed to re-check this image to ensure it accurately represented the points on the image. When I was satisfied I accepted the image. If it looked off-centred or flawed I would begin the process again. On accepting the image the pathways ™ program would import the image.

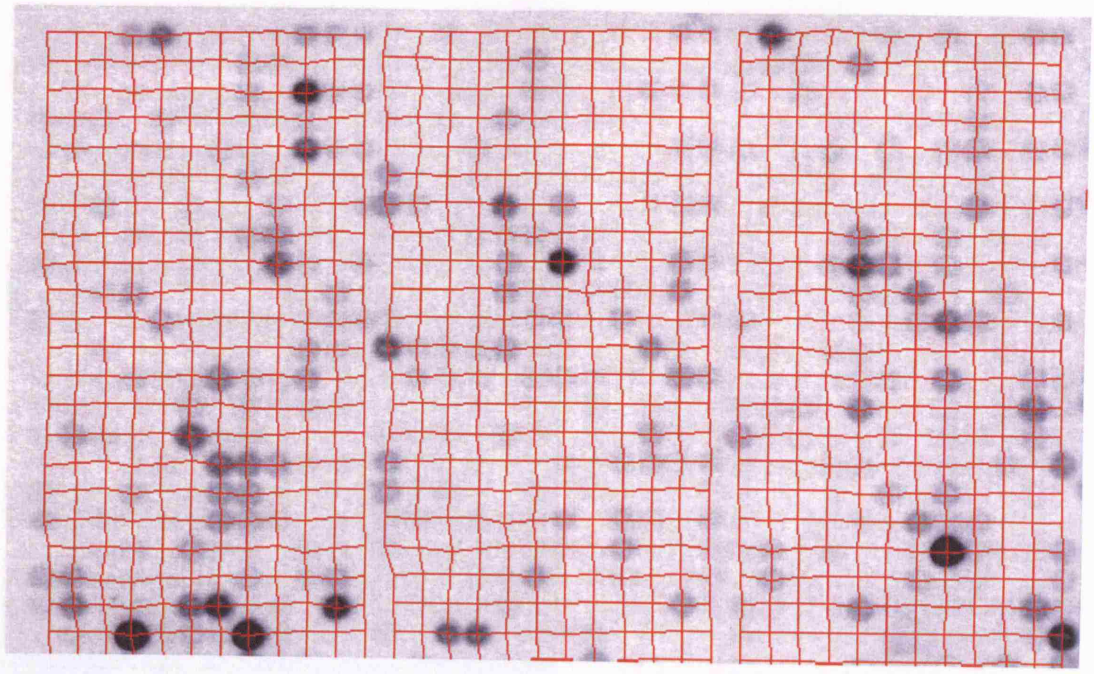


Figure 16 - Fine tuning the 16 point alignment

Example of fine tuning the 16 alignment points and grid created to ensure image is correctly aligned and imported

I imported all hybridised filters to the pathways ™ database. On each filter I performed a single GeneFilter analysis. This produced a synthetic or cleaned-up version of the original filter. All the spots appear the same size (with differing intensities), making analyses and comparisons simpler to comprehend. The image is compressed slightly to take up less space in the database. The synfilter display was an easy way of analysing the intensities of each gene on a grid. Figure 17 demonstrates the synfilter when comparing two images. By clicking on the reb/blue right hand panel, the gene of interest (in this case XRCC9) is demonstrated in the thumbnail. This is the image of each gene as it appears on the genefilter. All spots are shown on grid points allowing me to see exactly which gene is being expressed.

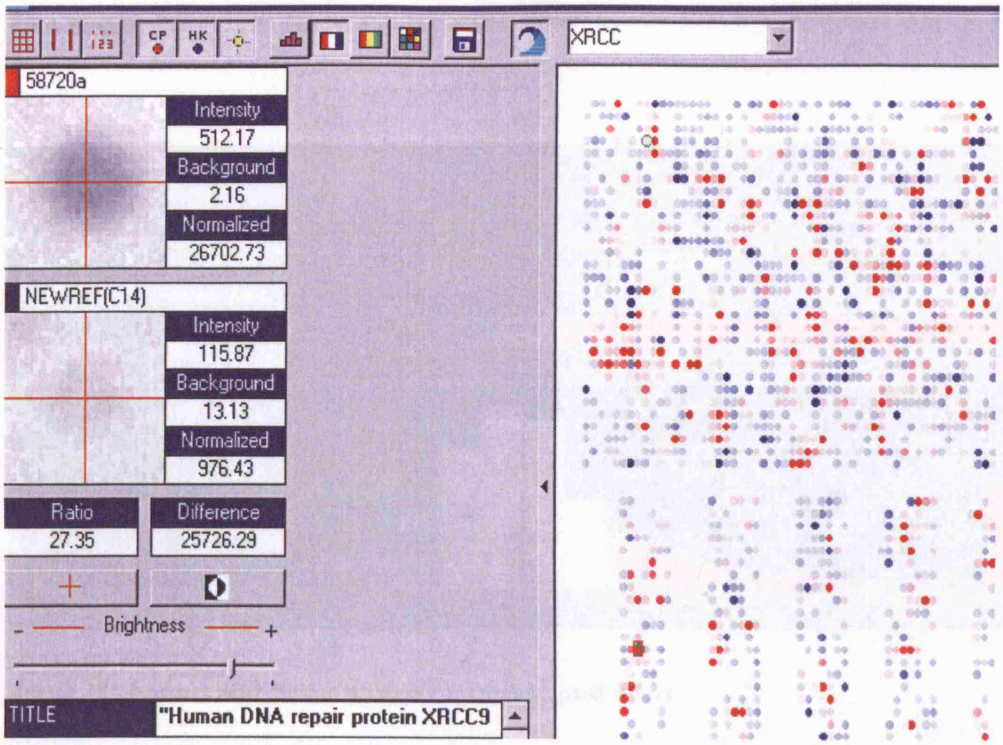


Figure 17 - Demonstration of pathways TM software toolbar (synfilter display)

The comparative filters are being displayed in the blue/red channel. High normalised intensity values are shown as red with a gradual decrease in the colour intensity through pink to white which indicates the expression in both samples for that gene is equivalent. Blue means a gene is underexpressed with again lightening shades of blue until white is reached. The thumbnail image shows the relative intensities of gene XRCC9 for both filters with a higher up fold expression in the order of 27 times for the top filter (58720 = post-720) when compared to the mixed reference.

3.7.2 Statistical Analysis

Using the Multiple GeneFilter Comparison feature of the pathways ™ software I compared each of the imported filters containing tumour samples with the mixed reference filter called New Ref C14. What I looked for was a normal distribution of

data points.

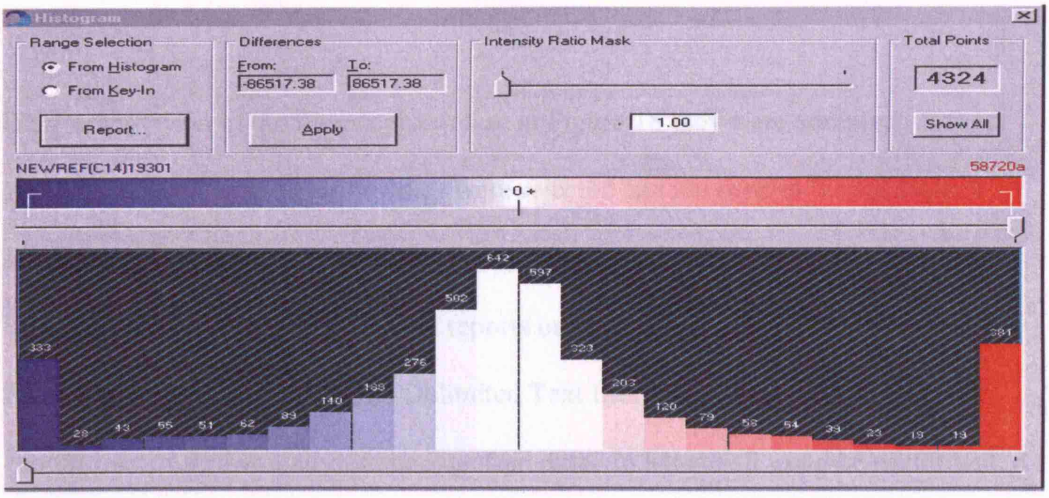


Figure 18 - Normal distribution analyses of two accepted filters

These filters were accepted for further analyses. This figure includes the values of sample post-720 compared to the reference sample. It demonstrates all values follow a normal distribution.

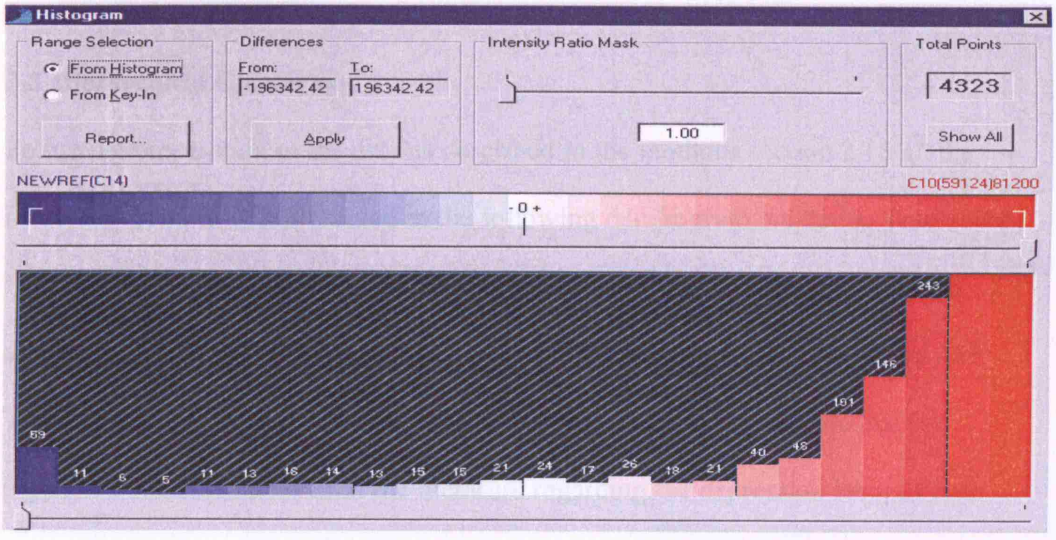


Figure 19 - Skewed distribution of a rejected filter

The data points on this filter (59124) were highly skewed compared to the reference sample, and was therefore not selected for further analyses.

If the comparison of the filters appeared as in Figure 18 they were accepted. If they appeared skewed as in Figure 19 they were rejected and not used in the subsequent analysis. On completing this process for all filters imported I then used the pathways ™ software to generate very detailed reports on each of the imported geneFilters. Each of these was saved as a Tab Delimited Text file. Dr Stephen Henderson our Bioinformatist further analysed the imported data. In Microsoft excel he performed the same statistical tests on each filter as described in the methods section 2.14. They included the mean, standard error, median, standard deviation, kurtosis and skewness. He established the minimum accepted dataset and selected the final genefilters for comparative analysis. This left me with a set of 16 tumour samples and two OS cell cultures.

3.8 Hierarchical Clustering

Following preparation of the data as described in the methods section 2.15 all the results are pictorially represented in the following dendrogram created in Treeview®. Figure 20 is the dendrogram of all the tumour samples demonstrating their gene expression of the retinoblastoma 1 gene, the retinoblastoma-like 1 (p107) and *p53*. The idea of selecting this section was to demonstrate that the data from this set supported what is known frpm the literature regarding the expression level of both retinoblastoma and p53 in the two commercial samples Saos-2 and MG-63.

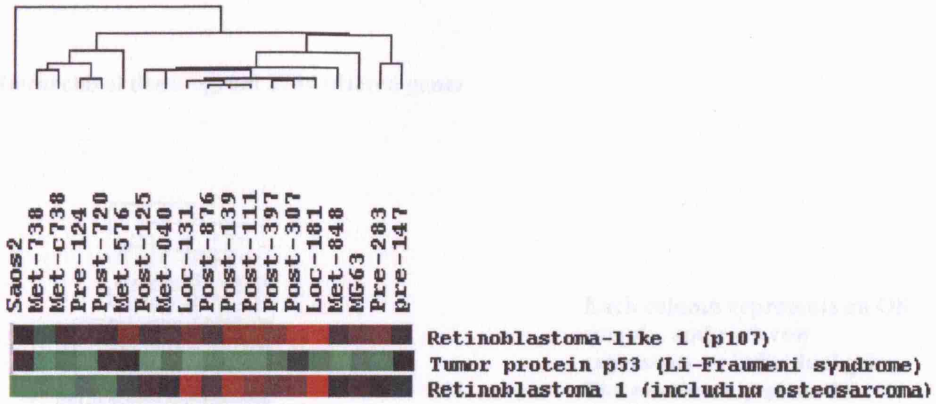
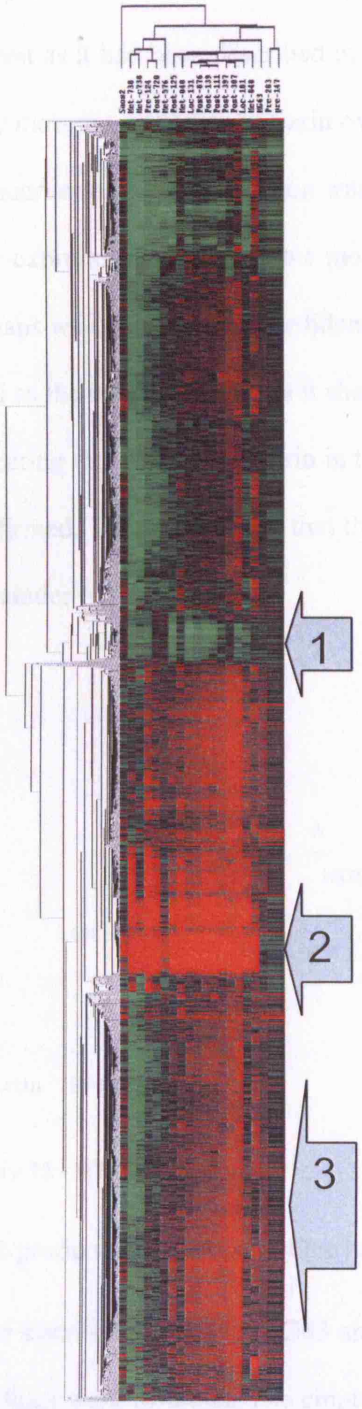


Figure 20 – Gene expression profile of p53 and pRb

Gene expression profile data showing expression level of p53 and Rb in Saos-2 and MG63 OS cell lines. This correlated with the known data that Saos-2 cells express p53 and are pRb deficient.

Figure 21 - Hierarchical dendrogram 2734 filtered genes



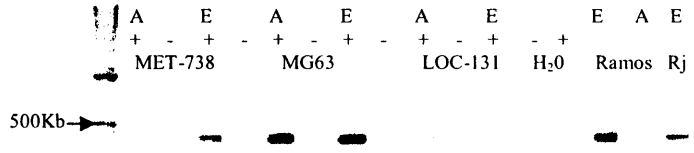
Each column represents an OS sample, and each row represents an individual gene. The relationship of the 16 samples plus two OS cell lines (MG63 & Saos2) is demonstrated at the top of the dendrogram. The shorter the branches the more similar the expression profile of either the genes or samples are to each other. Conversely the longer the branches the more dissimilar the expression profile. Arrow 1 shows a cluster of genes down regulated in OS samples compared to our reference probe, Arrow 2 shows the highly up-regulated genes and arrow 3 shows the region comprising the genes differentially expressed between the two main clusters A and B.

-10 **1** **+45**

Range of expression ratios correlating with colour intensity. Green reflects down regulation, red up regulation and black represents the same expression ratio as the reference sample

3.9 RT-PCR

The inclusion of results from RT-PCR performed on selected samples was to verify the results of the microarray experiments. Ezrin was selected as a gene of interest as it had been described in several publications as a protein involved with early metastatic potential. Ezrin over-expression was found in all our samples. As microarray gene expression was a new technology, if I could demonstrate the over-expression of ezrin with a more established technology such as RT-PCR perhaps we could be very confident with the rest of our novel findings. Actin was used as the control sample as it should be present in all cells. PCR products reflecting the presence of ezrin in tumour samples met-738 and loc-131 was confirmed. This data meant that there was no need to doubt the validity of the remainder of the dataset.



A=Actin E=Ezrin

Figure 22 - RT-PCR products

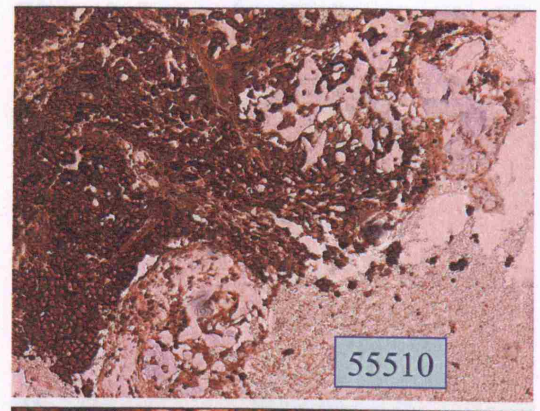
PCR products from ezrin (349bp) and actin (400bp) primers from three samples Met-738, MG63 and loc-131. Two control samples Ramos and Raji cell lines were included. The empty lanes in between samples show the PCR performed with cDNA made with no superscript added so demonstrating these are true PCR products from RNA and not contaminating DNA within the RNA sample. The

double band seen in Ramos cell DNA may reflect splice variants not present in the tumour cDNA.

3.10 Immunohistochemistry

Many tumours may overexpress a gene product but this not neccessarily mean its over-expression translates to increase production of the protein. As I had identified over-expression of ezrin as an interesting finding in my microarray data and subsequently confirmed this as "real" with an established technique RT-PCR I next wanted to see if in any of the corresponding paraffin emebedded tumours samples over-expressed ezrin by means of immunohistochemical staining (IHC). With the help of Dr Pringle we were able to indentify stored paraffin samples of tumour samples pre-147, pre-283, post-125, post-139, loc-181, met-738 and met 040. What I have included below are the original slides for the primary tumour (55510) which was the primary tibial tumour of the pateint who replased with the rib metastases met-738 (60738). I have shown the OS samples at low and high power at 60x in an effort to demonstrate the pattern of the ezrin IHC staining. In the primary tibial tumour (55510) at high power 60x the brown pattern denoting ezrin positivity has both a membranous and cytoplasmic distribution. This same patient's metastasis sample met-738 (60738) at high power 60x demonstrates a differential cytoplasmic staining in the tumour. This is of course very limited data based on one patient but it is interesting there is a difference in the pattern of ezrin positivity comparing the primary with the metastatic sample

Figure 23 - Ezrin staining in primary OS



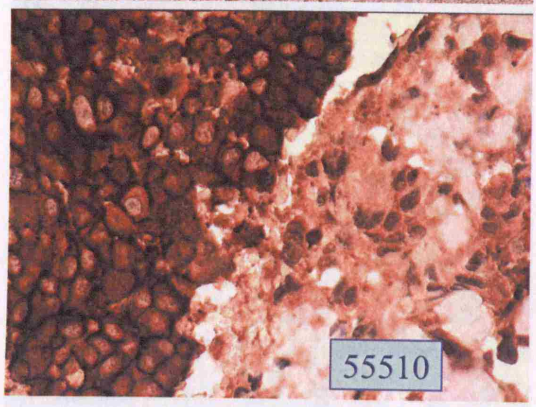
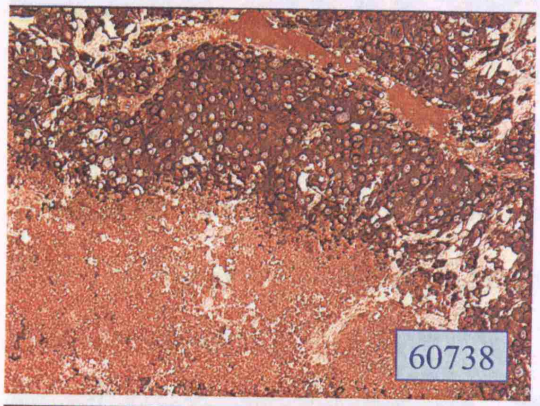


Figure 24 - Ezrin staining in metastatic OS



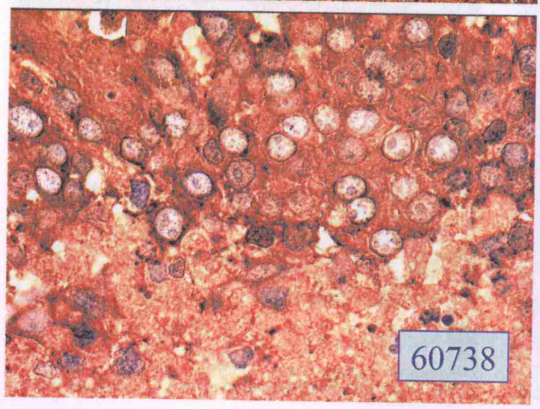
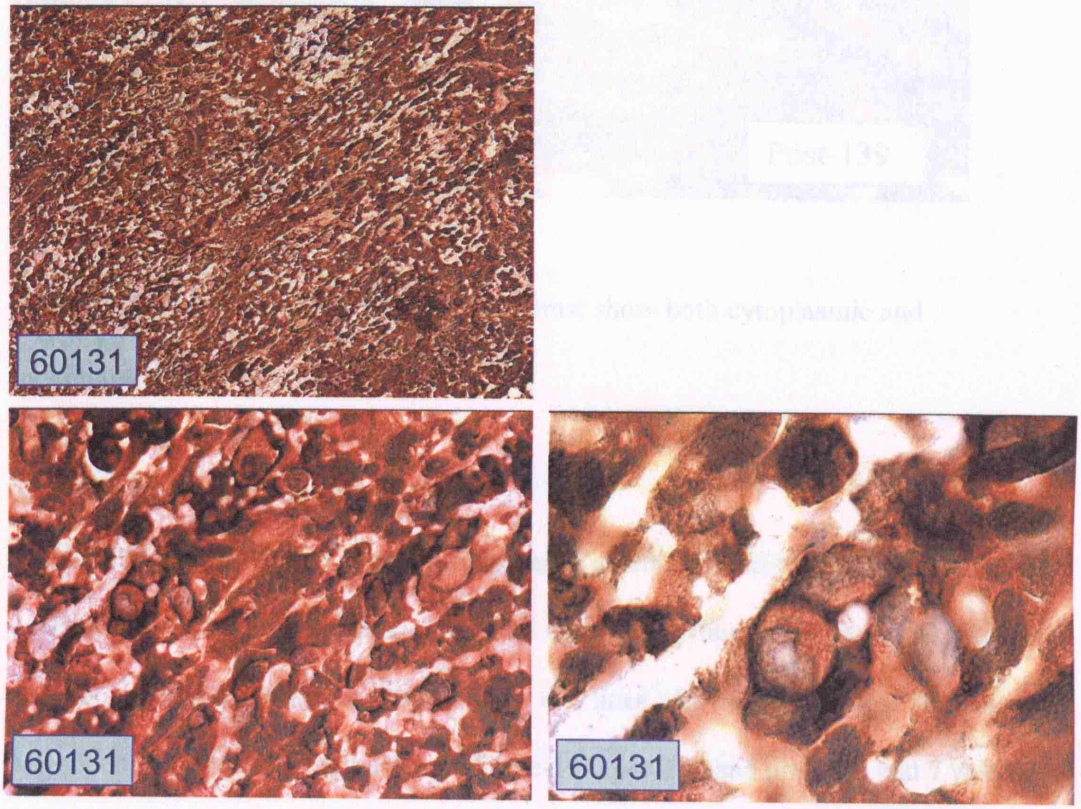
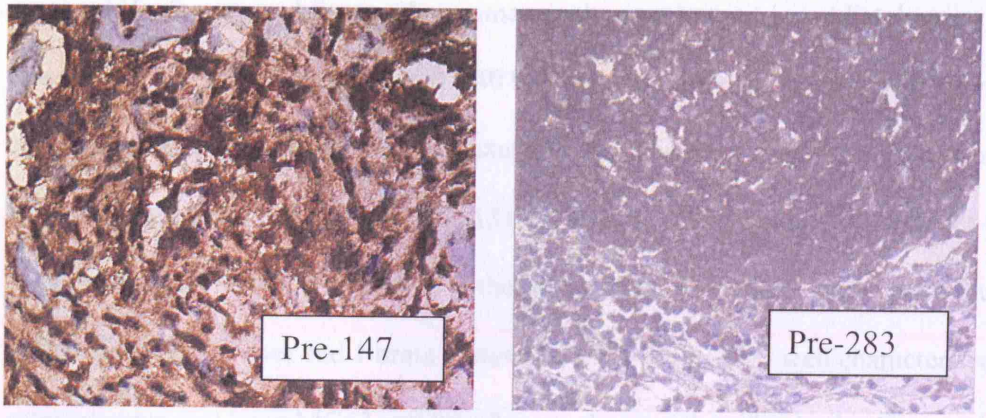


Figure 25 - Ezrin staining in all tumour samples



The above sample Loc-131 (60131) shows the pattern of ezrin expression in this local recurrence sample. At high power 60x there is evidence of both intense membranous as well as cytoplasmic staining. Two further corresponding primary samples (pre-147 & pre-283) display predominantly membranous staining. Both these samples are chemotherapy naive.



The two post-chemotherapy samples in contrast show both cytoplasmic and membranous staining

3.11 Gene expression profile data

To identify genes involved in different stages of sarcomagenesis I compared gene expression profiles from 16 tumour samples from both primary and metastatic tumours and 2 commercially available osteosarcoma cell lines. Of the 16 samples: three were diagnostic chemotherapy naïve samples (pre-124, pre-283 and pre-147) and 7 were post-chemotherapy resection samples (post-720, post-125, post-876, post139, post-397, post-111 and post-307). All samples were poor responders and were graded as having undergone less than 90% necrosis. The range was between 50% and <90%. Table 3 indicates the degree of necrosis of each of the post resection samples. The cytotoxic drugs used were doxorubicin/cisplatin in all regimens. Met-738 had also received Ifosfamide and Etoposide regimen in the past but not immediately prior to resection. The lung metastasis, met-740 had received the combination of Ifosfamide and etoposide prior to pulmonary metastatectomy. The remaining samples comprised: 2 local recurrences (Loc-181 and Loc-131), 2 lung metastases (met-576 and met-040), 1 rib metastasis (met-038, met-c038 is the probe made from the primary cell culture of the same tissue sample) and 1 brain metastasis (met-848). Two well characterised osteosarcoma cell lines MG63 and Saos2 were chosen. Saos 2 is both p53 and Rb null

and MG63 carries a mutated p53, but normal Rb [248, 249]. These were used as an internal control for the gene expression profile of our osteosarcoma tissues. Figure 20 shows that the microarray findings correlate with the published data. 2739 gene expression ratios for each tumour sample were obtained for analysis after excluding noise. All ratios were manually confirmed using each cDNA hybridisation image. The data set of 18 arrays each comprising 2739 filtered genes are represented pictorially in Figure 21. The hierarchical cluster tree on the left demonstrates the relationship each of the genes has to each other. The shorter the branches the more similar their expression profiles. The longer the branches, the more dissimilar the relationship. Functionally similar genes cluster together. Up regulated genes are shown in red, down regulated genes are shown in green and those that have similar expression values to the reference sample are shown in black. The varying colour intensity of either green or red reflects how up or down regulated the expression ratios are. Figure 21 shows the final clustering of the samples and their relationship to each other. The dendrogram at the top of the Treeview® image shows the relationship the patient samples and osteosarcoma cell lines have to each other. An enlarged version is shown below in Figure 26.

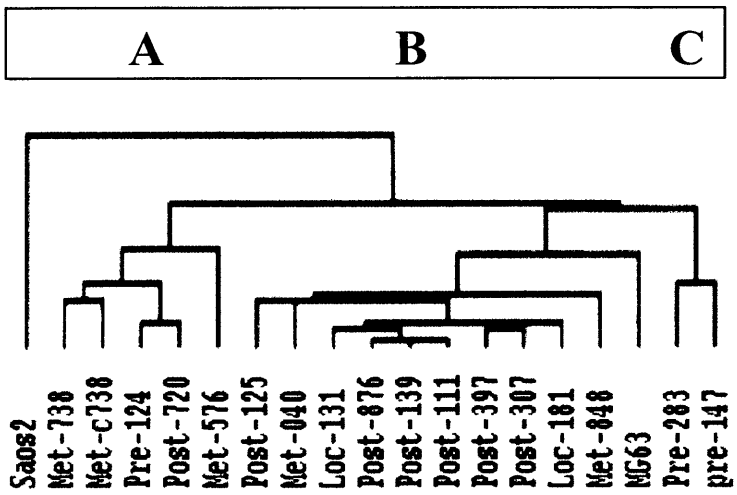


Figure 26 – The final clustering of samples and cell lines

Cluster A contains 5 samples, 4 of which had not received any immediate prior cytotoxic chemotherapy. The remaining sample post-720 had received only one cycle of chemotherapy but their tumour grew through treatment and the specimen showed no necrosis. The main cluster, B which contains 10 samples comprises predominantly specimens which underwent immediate chemotherapy prior to resection. Although the samples removed were taken from the viable tumour remaining post resection, the final histological report states varying degrees of histological damage. Cluster C comprises only two pre-treatment primary extremity osteosarcoma samples. The osteosarcoma cell line MG63 clusters most closely with cluster B. This cell line was derived from a 14 year old male and in addition to its Rb and p53 status expresses receptor for TGFβ. The profile of Saos 2 remains outside all clusters and appears the most distinct. Saos 2 are not tumorigenic in immunosuppressed mice but do form colonies in semi-solid medium [250]. They are said to have low metastatic potential in vivo [173, 251, 252] and are multidrug resistant [253]. They were cultured from an 11 year old female post-chemotherapy. Many variants of this cell line with metastatic potential and increased drug resistance have since been developed [166, 254].

The red region seen across all samples reflects 264 genes significantly up-regulated from the reference sample (Figure 21 Section 2). Table 6 shows the genes with a greater than 10 fold increase as compared with the reference sample. Dr Stephen Henderson performed further statistical analysis on the whole dataset and table 7 shows the most strongly expressed genes within the patient tumour samples (above the 99^{th} percentile of mean expression). This contains many known bone markers: in particular, Collagen-1,alpha-2 and Matrix Gla which are all major constituents of bone matrix. All of these were high in all samples demonstrating the high osteoid content of all patient samples.

Acc	Name	Mean level of expression
AA664180	glutathione peroxidase 3 (plasma)	45
AA707922	phosphodiesterase 6H, cGMP-specific, cone, gamma	40
N71628	**Spi-B transcription factor (Spi-1/PU.1 related)**	**36**
AA436564	Human cellular proto-oncogene (c-mer) mRNA	35
N91385	membrane-spanning 4-domains, subfamily A, member 1	35
AA411440	**villin 2 (ezrin)**	**34**
N78621	adaptor-related protein complex 1, gamma 1 subunit	33
N46828	inositol 1,4,5-trisphosphate 3-kinase C	33
AA281635	interleukin 24	33
AA620859	**sarcospan (Kras oncogene-associated gene)**	33
AA150828	**mitogen-activated protein kinase kinase kinase 5**	33
AA457155	zinc finger protein 212	31
AA397824	D-dopachrome tautomerase	30
AA427934	**Rho GTPase activating protein 1**	**30**
AA046523	centrin, EF-hand protein, 3 (CDC31 yeast homolog)	29
R56211	platelet-derived growth factor receptor, beta polypeptide	29
AA481758	DnaJ (Hsp40) homolog, subfamily B, member 1	29
AA778392	BENE protein	29
AA419177	solute carrier family 7	29
W37753	ceroid-lipofuscinosis	28
AA633901	**transforming growth factor, beta-induced, 68kD**	**28**
AA699469	carbonic anhydrase VA, mitochondrial	27
AA629923	pM5 protein	25
AA235706	TAF10 RNA polymerase II	25
AA872001	annexin A6	23
AA046690	kinesin family member 5B	23
H94857	GCN5	23
AA076063	**Caldesmon**	**22**
AA292536	cut (Drosophila)-like 1 (CCAAT displacement protein)	20
H48494	glucosamine-6-phosphate isomerase	20
AA129537	**Ras-GTPase-activating protein SH3-domain-binding protein**	**19**
AA460830	polymerase (RNA) II	19
AA668681	**cell division cycle 42**	**18**
AA280846	carbonyl reductase 1	17
AA461527	COP9	17
AA477428	polymerase (RNA) II	16
AA425299	**Homo sapiens ezrin-radixin-moesin binding phosphoprotein**	**15**
AA489383	**bone morphogenetic protein 2**	**15**
AA521431	**profilin 1**	**12**
AA029964	ataxin 2 related protein	12
AA155913	**matrix Gla protein**	**11**

Table 6 - The most upregulated genes

Accession number	Gene name
AA076063	Caldesmon
AA397824	Dopachrome tautomerase
AA521431	Profilin
AA155913	MatrixGla protein
AA872001	Annexin VI
AA490172	Collagen-1,ά-2
AA411440	Ezrin

Table 7 - The statistically significant over expressed genes

Another highly expressed gene, ezrin, lay just below the 99th percentile of expression and is reported for the first time in primary OS samples. This gene is of particular interest due to its previously reported association of metastasis in an OS xenograft model [252]. All samples in this dataset demonstrated a high expression of ezrin. Ezrin has been identified as a key component of paediatric cancer metastasis. It is a member of a family of closely related intracellular proteins, the *ERM* family (named for its first three members, ezrin, radixin, and moesin), which acts to attach actin filaments to the plasma membrane in many cell types. The C-terminal domain of ERM proteins binds directly to the sides of actin filaments. The N-terminal domain binds to the cytoplasmic face of several transmembrane glycoproteins, including CD44, the receptor for the extracellular matrix component hyaluronan. The functional importance of the ERM proteins is indicated by the consequences of mutations that lead to a loss of one of the members of the family, called merlin. Merlin is associated with cell-adhesion function and has been proposed to compete with ezrin for CD44-complex function. The loss of Merlin would be equivalent to the up-regulation of ezrin [255]. Unlike the attachments between actin and the plasma membrane mediated by spectrin or myosin I, those mediated by ERM proteins are regulated by both intracellular and extracellular signals. ERM proteins can exist in two conformations, an active extended conformation that oligomerises and binds actin and CD44, and an

inactive folded conformation, in which the N- and C-termini are held together by an intramolecular interaction. Switching between the two conformations can be triggered by phosphorylation or binding to Phosphatidyinositol 4-5 biphosphate (PIP_2), either of which can occur, for example, in response to extracellular signals. Thus, the strength of ERM-mediated contacts between the actin cytoskeleton and the extracellular matrix is sensitive to a variety of signals received by the cell [7]. Since their original publication Khanna et al [252] have since elucidated some of the signal transduction pathways they propose are involved in developing metastases in OS and rhabdomyosarcoma (RMS) [256-258]. Their work has been performed in both murine and dog OS but infer the results can be transferable to human OS. In their study, they found that a mechanism of ezrin-related metastatic behavior is linked to an Akt-dependent mammalian target of rapamycin (mTOR)/p70 ribosomal protein S6 kinase (S6K1)/eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) pathway. Blocking the mTOR pathway with rapamycin led to significant inhibition of experimental lung metastases in vivo. These results suggest that blocking the mTOR/S6K1/4E-BP1 pathway may be an appropriate target for strategies to reduce tumor cell metastasis [256-258]. Ezrin has been implicated in many roles, for example, as a conduit for signals between metastasis-associated cell-surface molecules and signal transduction components. This suggests that ezrin and, potentially, other members of the ERM (ezrin-radixin-moesin) family have key roles in the coordination of signals and cellular complexes that are required for the successful metastasis of these and other malignancies [259]. Although there was no difference in the level of expression across the OS samples in my study, I think reflected the similarity of all the samples coming as they did from patients with a poor prognosis. IHC staining of just a few corresponding samples was included only to verify the gene expression of ezrin was translated into subsequent protein expression. The differential staining pattern of a

distinctive cytoplasmic pattern in the metastatic sample compared to both membrane and cytosolic staining seen in the original primary and separate local recurrence does pose an interesting question for future work. This differential staining pattern of ezrin has been reported in uterine endometrial carcinoma (UEC). Ohtani et al reported a study employing immunohistochemistry (IHC) in UEC. They found ezrin was localized in the membrane of metastasised cancer cells, when ezrin was mainly distributed in the cytoplasm of most other cancer cells and some endometrial hyperplastic cells. On performing Western blot analysis, ezrin was also detected in both cytosolic and membrane fractions in atypical endometrial hyperplasias (aH) and UEC, whereas ezrin was detected only in the cytosolic fraction in simple endometrial hyperplasias (sH) and complex endometrial hyperplasias (cH). Ohtani's data suggest that expression and subcellular distribution of ezrin protein play an important role in the development and progression of UEC [260]. Other genes significantly highly expressed include Caldesmon and Profilin. Both of these genes are involved in binding to actin and affecting the structure of the cytoskeleton hence their association with ezrin.

Other genes over-expressed in the red area include COP9 and cell division cycle 42. Both these genes are involved in signal transduction. Cell division cycle 42 is a small GTPase of the Rho-subfamily, which regulates signalling pathways that control diverse cellular functions including cell morphology, migration, endocytosis and cell cycle progression. This protein could regulate actin polymerisation through its direct binding to Neural Wiskott-Aldrich syndrome protein (N-WASP), which subsequently activates Arp2/3 complex. The protein encoded by gene COP9 is one of the eight subunits of COP9 signalosome, a highly conserved protein complex that functions as an important regulator in multiple signaling pathways. The structure and function of COP9 signalosome is similar to that of the 19S regulatory particle of 26S proteasome.

COP9 signalosome has been shown to interact with SCF-type E3 ubiquitin ligases and act as a positive regulator of E3 ubiquitin ligases. This protein is reported to be involved in the degradation of cyclin-dependent kinase inhibitor CDKN1B/p27Kip1. It is also known to be an coactivator that increases the specificity of JUN/AP1 transcription factors [261].

Transforming growth factor beta, Interleukin 24, Spi-B transcription factor and Annexin VI are genes commonly expressed by all OS samples in this research. TGFβ binds to type I, II, and IV collagens. This adhesion protein may play an important role in cell-collagen interactions in cartilage, and may be involved in endochondral bone formation [261]. Spi-B transcription factor binds to the pu-box, a purine-rich DNA sequence (5'- gaggaa-3') that can act as a lymphoid-specific enhancer. It might affect spi-b function by recruiting factors involved in spi-b activity [261]. Interleukin 24 is a gene which encodes a member of the IL10 family of cytokines. It was identified as a gene induced during terminal differentiation in melanoma cells. The protein encoded by this gene can induce apoptosis selectively in various cancer cells. Overexpression of this gene leads to elevated expression of several GADD family genes, which correlates with the induction of apoptosis. The phosphorylation of mitogen-activated protein kinase 14 (MAPK7/P38), and heat shock 27kDa protein 1 (HSPB2/HSP27) are found to be induced by this gene in melanoma cells, but not in normal immortal melanocytes. Alternatively spliced transcript variants encoding distinct isoforms have been reported [261]. Annexin VI belongs to a family of calcium-dependent membrane and phospholipid binding proteins. Although their functions are still not clearly defined, several members of the annexin family have been implicated in membrane-related events along exocytotic and endocytotic pathways. Annexin VI has been implicated in mediating the endosome aggregation and vesicle fusion in secreting

epithelia. It may associate with cd21 and may regulate the release of Ca^{2+} from intracellular stores.

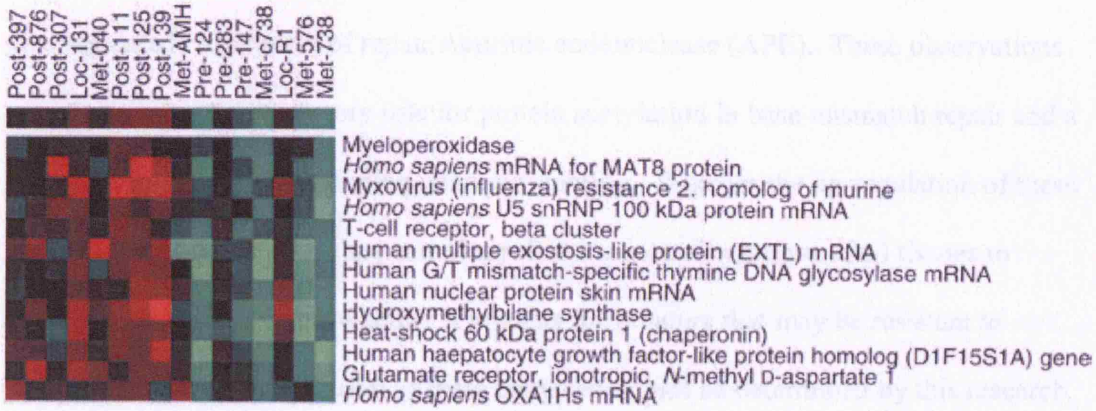


Figure 27 - Chemotherapy heatmap

Figure 27 illustrates genes highly expressed in chemotherapy treated patients immediately prior to resection. All differences were significant at $p<0.01$. This group of differentially expressed genes was coined the chemotherapy heatmap as a number of upregulated genes in this group are associated with detoxification. Myeloperoxidase (MPO) is part of the host defence system of human polymorphonuclear leukocytes (PMN). In the stimulated PMN, MPO, reacts with the H_2O_2 formed by the respiratory burst to form a complex that can oxidize a large variety of substances [262]. In this scenario intranuclear MPO may have been released by the invading PMN in the OS samples to help protect DNA against damage resulting from oxygen radicals produced during the cytotoxic chemotherapy. The associated presence of other highly expressed genes whose function is also to protect against DNA damage would fit with this explanation. Thymine DNA glycosylase (TDG) initiates repair of G/T and G/U mismatches, commonly associated with CpG islands, by removing thymine and uracil moieties [263, 264]. TDG associates with transcriptional coactivators CRE-binding

protein (CBP) and p300 [264] and the resulting complexes are competent for both the excision step of repair and histone acetylation. TDG stimulated CBP transcriptional activity in transfected cells and reciprocally served as a substrate for CBP/p300 acetylation. This acetylation triggered release of CBP from DNA ternary complexes and also regulated recruitment of repair Apurinic endonuclease (APE). These observations revealed a potential regulatory role for protein acetylation in base mismatch repair and a role for CBP/p300 in maintaining genomic stability. Whether the up-regulation of these genes in the post chemotherapy samples reflects a normal response of all tissues to cytotoxic chemotherapy or whether it predicts for tumours that may be resistant to chemotherapy by up-regulation of these enzymes cannot be determined by this research.

The up-regulation of XRCC9 and XRCC4 in both the post-chemotherapy samples which had a poor response to treatment and in the recurrent tumours may be more indicative of inherent chemoresistance of these OS samples. Both these genes are nucleotide excision repair (NER) genes. XRCC9 is markedly elevated compared to the reference samples. It was characterised by Lui et al in 1997 and found to partially correct mutagen sensitivity and chromosomal instability in UV40 cells up-regulation [265]. XRCC9 is described as a candidate tumor suppressor gene that might operate in a postreplication repair or a cell cycle checkpoint function [265]. XRCC4 gene restores DNA double-strand break repair and the ability to support variable (V), diversity (D), and joining (J) recombination of transiently introduced substrates in the XR-1 CHO cell line [266, 267]. Whether the up-regulation of these genes represents a select class of DNA repair genes which protect OS from cisplatinum damage needs further research. The well described chemoresistant gene p-glycoprotein 3 (Pgp) is also shown in Table 8 for comparison. Increased levels of p-glycoprotein are found in many cancer cells and increased levels are detected after cytotoxic chemotherapy particularly when the tumour has become refractory to treatment [215]. In OS p-

glycoprotein expression has been extensively studied. There are inconsistencies in some of the results of the various studies trying to elucidate its role but the consensus from the available evidence showed conclusively that p-glycoprotein was not associated with the histological response of patients with OS to combination chemotherapy regimens, but conversely, Pgp positivity, as determined by immunohistochemistry, was a strong correlate of more rapid disease progression. It is acknowledged that although there was heterogeneity across the performed studies p-glycoprotein is a marker of poor outcome and drug resistance [216, 268]. In this work p-glycoprotein was not over-expressed in any of the OS samples. Only four out of the sixteen samples used in this work were chemonaive with the reminder being exposed to cytotoxic chemotherapy either in the previous two to three weeks or within the last two years. Six of the samples represent recurrent disease and in none of those samples was an elevated level of p-glycoprotein detected.

Gene name	Mean level of expression in Cluster A	Mean level of expression in Cluster B	Mean level of expression in Cluster C
Human DNA repair protein XRCC9	28.03	39.84	-1.61
DNA repair protein XRCC4	2.37	3.33	1.5
DNA excision repair protein ERCC5	-1.51	3.19	1.33
DNA mismatch repair protein MLH1	-1.06	2.52	1.00
P glycoprotein 3/multiple drug resistance 3	-1.23	-1.41	-1.05

Table 8 – Genes associated with DNA repair to cytoxic chemotherapy

Genes associated with DNA repair to cytotoxic chemotherapy damage and their level of expression found in our samples.

The nucleotide excision repair genes (NER) XRCC9 and XRCC4 were found to be elevated in all relapsed or chemotherapy treated samples compared to the two samples

which had not received any chemotherapy. P-glycoprotein expression was not elevated in any samples.

The rearrangement T-cell receptor, beta cluster gene was found to be present in a study of lymphoproliferative disease, all 6 T-cell leukaemias and in 16 of 19 T-cell lymphomas [269]. It is interesting therefore this gene was overexpressed in the cluster which had received prior chemotherapy. Is its presence a marker of lymphocyte recruitment during cell damage and necrosis or may it have a role in the pathogenesis or susceptibility to developing OS. Three of the patients who developed OS in this cohort were survivors of ALL. This does seem an unusually high number of patients in such a small sample that each had a second malignancy of OS following successful treatment of ALL. This is a well described phenomena [270]. Such individuals may belong to Li Fraumeni families but I have no further history or data to support this. The existence of other genetic susceptibility caused by gene rearrangements such as t(1;19) usually seen in B precursor cell ALL and del 13 found in the great majority of OS have been studied but no association has so far been found [271]. I do not have the details of how their ALL was managed but in addition to the presumption of genetic susceptibility must be the influence of the treatment they each would have received including cytotoxic chemotherapy and possibly a bone marrow transplant. I cannot therefore draw any further conclusions from their inclusion in my data.

Molecular chaperones are a ubiquitous family of cellular proteins which mediate the correct folding of other polypeptides but are not components of those final structures. Chaperones function by binding specifically and non-covalently to interactive protein surfaces that are exposed transiently during cellular processes such as protein synthesis, protein transport across membranes, DNA synthesis, the recycling of clathrin cages, the assembly of organellar complexes from imported subunits, and stress responses. Heat shock 60 kDa protein 1 (chaperonin) is a member of this family

of proteins [272]. The explanation for its up-regulation in the post-chemotherapy samples compared to those not immediately exposed to cytotoxic treatment may just reflect increased cellular activity.

Mat-8 is a novel 8-kDa transmembrane protein that is expressed in a subset of murine breast tumors. The extracellular and transmembrane domains of Mat-8 are homologous to those of phospholemman (PLM), the major plasmalemmal substrate for cAMP-dependent protein kinase and protein kinase C in several different tissues. It is thought that Mat-8 and PLM, the products of distinct genes, are related proteins that serve as Cl- channels or Cl- channel regulators but have different roles in cell and organ physiology. Whether its up-regulation here suggests a role in chemotherapy transport or detoxification or perhaps a novel finding in OS cannot be determined from this small study.

Acc	Name	p value
AA156988	aconitase 1, soluble	2.58E-05
AA670422	ADP-ribosylation factor 3	0.000208
H18070	mitochondrial translational initiation factor 2	0.000261
R40324	clones 23667 and 23775 zinc finger protein	0.000268
AA055350	adenosine A2b receptor	0.000304
W45572	ADP-ribosylation factor 1	0.000349
AA454146	Cyclin H	0.000385
AA425853	splicing factor proline/glutamine rich	0.000444
N56693	**cytochrome c oxidase subunit VIIb**	**0.000446**
AA464147	cysteinyl-tRNA synthetase	0.000506
R12473	adenosine kinase	0.000535
AA070358	transketolase (Wernicke-Korsakoff syndrome)	0.000581
AA280647	signal transducer and activator of transcription	0.00062
AA461157	neural cell adhesion molecule 1	0.000687
AA598561	**CD164 antigen, sialomucin**	**0.000764**
H07899	**vascular endothelial growth factor C**	**0.000807**
H68838	nuclear receptor subfamily	0.000843
AA448277	forkhead box O1A (rhabdomyosarcoma)	0.000894
AA256419	insulin-like growth factor 1 receptor	0.000932
R46816	**aldehyde dehydrogenase 9 family**	**0.000974**
H20743	cell division cycle 34	0.001012
AA136882	activin A receptor, type I	0.001051
AA453776	alcohol dehydrogenase 5	0.001099
AA418694	ATX1 (antioxidant protein 1, yeast)	0.001272
AA457547	eukaryotic translation initiation factor 4 gamma	0.001279
R91950	**cytochrome b-5**	**0.001288**
T82414	**RAB2, member RAS oncogene family**	**0.001359**
AA127100	ribophorin I	0.00141
AA496947	thymine-DNA glycosylase	0.00151
H69335	Pirin	0.001533
H15112	**uracil-DNA glycosylase**	**0.001826**
AA775872	**glypican 3**	**0.002035**
AA136271	**CD58 antigen**	**0.002632**
N51095	**rho family, small GTP binding protein Rac3**	**0.003331**
AA417881	**bleomycin hydrolase**	**0.003355**

Table 9 - 35 most differentially expressed genes between cluster A&B

Figure 21, demonstrates the down regulated genes which have functionally clustered together. Some of these genes included the expression of CD68 an antigen marker of macrophages/histiocytes, vimentin a marker for fibroblasts, CD31 a platelet/endothelial cell marker and keratin 18 an epithelial marker. This supports the use of the mixed reference sample in screening out for such contaminants and therefore leaving only the genes significant to the tumour itself. The tumour

suppressor gene p53 (Li Fraumeni) is amongst this group as is another tumour suppressor Caveolin 1.

Acc	Title	Mean fold down Expression
AA879933	Human extracellular protein (S1-5) mRNA	10.82
AA423867	Human prepromultimerin mRNA	7.03
AA486321	**Vimentin**	**4.08**
H11003	**Endothelin 1 {alternative products}**	**3.77**
AA490477	Smooth muscle myosin heavy chain isoform SMemb	3.22
N98412	Human mRNA for HHR23A protein	3.16
T60048	Gamma-actin, enteric smooth muscle form	2.66
AA701981	Human C2f mRNA	2.58
AA278759	Hematopoetic proteoglycan core protein	2.54
AA411343	Ribosomal protein S29	2.40
AA664179	**Keratin 18**	**2.39**
AA127014	Human mitochondrial NADH dehydrogenase-ubiquinone Fe-S protein 8	2.26
AA410517	Cytoplasmic antiproteinase	2.22
AA495790	**ras homolog gene family, member B**	**2.13**
AA486741	Argininosuccinate lyase	2.09
R26982	Homo sapiens HKL1 mRNA	2.08
N63943	Lysozyme	2.00
AA669359	Ribosomal protein L44	2.00
AA486393	**Interleukin 10 receptor, beta**	**1.99**
N59893	Human BRCA2 region mRNA	1.97
AA634008	40S RIBOSOMAL PROTEIN S23	1.96
N57964	Chemokine (C-C motif) receptor 6	1.89
AA485922	Human copine I mRNA	1.89
R39356	**Tumor protein p53 (Li-Fraumeni syndrome)**	**1.88**
AA010605	4-hydroxyphenylpyruvate dioxygenase	1.87
R37286	Human hnRNP core protein A1	1.87
AA598794	Connective tissue growth factor	1.87
R87642	60S RIBOSOMAL PROTEIN L34	1.85
R76263	Human HS1 binding protein HAX-1 mRNA	1.85
AA477781	Cytochrome P450, 51 (lanosterol 14-alpha-demethylase)	1.83
AA055835	**Caveolin, caveolae protein, 22kD**	**1.82**
AA640040	TRYPTOPHANYL-TRNA SYNTHETASE	1.81
AA629897	Laminin receptor (2H5 epitope)	1.80
AA489201	H.sapiens mRNA for PHAPI2b protein	1.79
H61243	Uncoupling protein 2 (mitochondrial, proton carrier)	1.79
AA292226	Homo sapiens creatine transporter mRNA	1.78
AA706987	H.sapiens mRNA for UDP-GalNAc:polypeptide	1.77
AA878561	Ubiquitin A-52 residue ribosomal protein fusion product 1	1.766
R62817	ERYTHROCYTE BAND 7 INTEGRAL MEMBRANE PROTEIN	1.76
W04674	Homo sapiens mRNA for cytochrome b5	1.75
H9778	**H.sapiens mRNA for E-cadherin**	**1.74**

Table 10 - The most down regulated genes

In the hierarchical clustering of all samples (Figure 26) met-738 and the primary cell culture, I established from this lesion (met-c738), clustered tightly together. This suggests that for array analysis, early passage primary cell culture, rather than laser capture microdissection (LCM) [273], could be useful to enrich for OS cells from a mixed tumour stromal microenvironment. This was fortunate as it was not technically possible to employ LCM on OS samples.

An analysis of genes differentially expressed between primary and metastatic samples is shown in Figure 28.

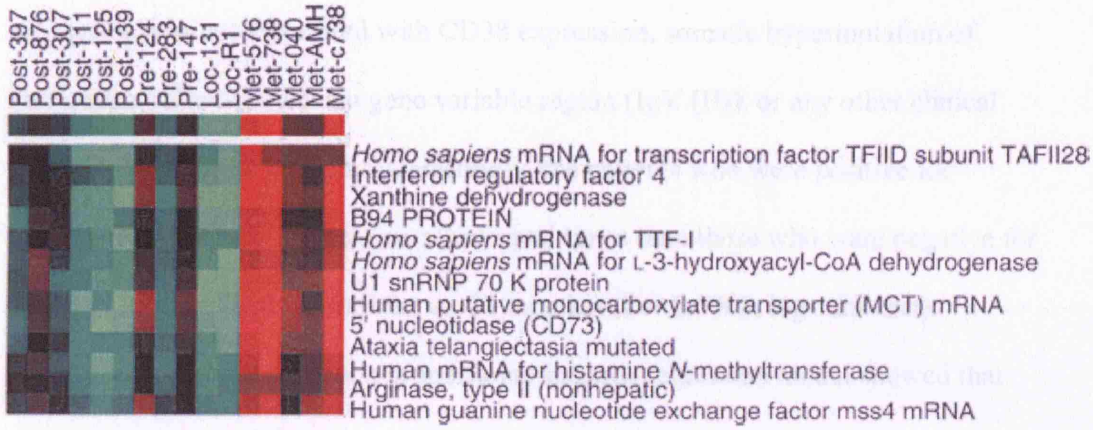


Figure 28 - Metastatic heatmap

Figure 28 shows genes that are highly expressed in metastatic samples ($p<0.01$).

Among the genes highly expressed in the metastatic samples was the powerful anti-oxidant xanthine dehyrogenase, interferon regulatory factor- 4 and CD 73 a gene associated with lymphocyte activation. Xu et al described xanthine dehydrogenase as belonging to the group of molybdenum-containing hydroxylases involved in the oxidative metabolism of purines [274]. Xanthine dehydrogenase (XDH) is a rate-limiting enzyme in the oxidative metabolism of purines and is thought to play a key role in a variety of pathophysiological processes including ischemia solid reperfusion

injury, viral pneumonia, and renal failure. MUM1 (for 'multiple myeloma oncogene 1') gene, is also referred to as interferon regulatory factor-4 (IRF4), a member of a gene family known to be active in the control of B-cell proliferation and differentiation and was also over-expressed in the metastatic OS samples compared to the primaries. MUM1/IRF4 is a proto-oncogene that is deregulated as a result of the t(6;14) (p25;q32) chromosomal translocation in multiple myeloma, and is also expressed in a variety of malignant lymphoma entities. Iida et al examined the expression of MUM1 in B-CLL and found that 2 of 4 B-CLL-derived cell lines and 14 of 29 patients' specimens expressed MUM1/IRF4 by immunohistochemical analysis. MUM1/IRF4 expression was not associated with CD38 expression, somatic hypermutation of immunoglobulin heavy chain gene variable region (IgV (H)), or any other clinical characteristics of the patients. Interestingly, the patients who were positive for MUM1/IRF4 showed shorter overall survival times than those who were negative for MUM1 (50% survival: 22 months vs. 82 months) ($P = 0.0008$, log-rank test). Multivariate analysis by Cox's proportional-hazards regression model showed that MUM1 expression and unmutated IgV (H) status were independent unfavourable prognostic factors in patients with B-CLL. These findings suggest that MUM1/IRF4 expression is a useful prognostic factor in B-CLL. The biological role and mechanism of action of MUM1/IRF4 in these metastatic samples of OS needs to be clarified [275, 276]. The fact there is increased expression in the metastatic as opposed to the primary samples would fit as these patients who have relapsed are in a poor prognostic group.

Both local recurrence samples had a profile more closely related to primary rather than metastatic disease Figure 26.

Cluster C contains only two patient samples. They are both pre-treatment primaries and one of which represents data obtained from a needle core biopsy. Core biopsies are taken under ultrasound guidance and could be said to represent pure tumour populations as they are taken from the centre of the tumour with very little supporting stromal tissue. The size and the distribution of genes within a tumour have been looked at by other groups. Small tumours (3-4mm in diameter) express more bFGF and IL-8 than large tumours (>10mm), whereas more VEGF is expressed in large tumours. Immunostaining shows a heterogeneous distribution of angiogenic factors within a tumour; the expression of bFGF and IL-8 was highest on the periphery of a large tumour where cell division is maximum. VEGF expression was higher in the centre of a tumour [277, 278]. This is particularly true in the strongly up regulated section. In all the significant up regulated genes, only a few genes e.g. ezrin show similar levels of expression between the samples in cluster A and the rest. Perhaps we can infer for these genes that their presence is more evenly expressed throughout the whole tumour.

Chapter 4 Discussion and Summary

Discussion

- I have successfully cultured and maintained primary OS cells from a fresh OS tumour

- I have successfully extracted RNA from both fresh OS tissue and established cell lines and primary OS cultured cells

- I have demonstrated that it is feasible to extract enough RNA from both diagnostic needle core and excision biopsy tissue samples to generate cDNA probes for microarray analysis

- I have used the extracted RNA to generate cDNA probes for OS tumour samples

- I have obtained gene expression microarray data on 16 OS samples.

- I have demonstrated that the OS samples in my samples cluster into two main groups: the chemotherapy naïve versus the post chemotherapy samples

- I have shown that tumours from the same individual more closely cluster together, than similar tumours from different individuals

- I have identified known and novel putative markers for OS that could have prognostic significance

Primary cell culture from fresh OS tissue

When the research project was first put together the use of Laser capture microdissection (LCM) was being employed for other tumour types prior to microarray analysis. OS is a bone forming tumour and the technology of LCM of capturing pure populations of OS was not available at that time. Instead if it was possible to produce a primary cell culture from a fresh OS tumour this could mimic LCM as only malignant cells could grow in a flask of media. By selecting the pure tumour cell population from which RNA could be extracted then the contamination from supporting stromal cells, endothelial cells, fat and invading lymphocytes could be avoided and the cDNA probes generated from this pure RNA OS population of cells could give us more precise insights about OS. I was able to demonstrate the ability to sustain a culture of malignant cells from several of the fresh OS samples I obtained. One sample met – 738 was fully characterised for morphology by electron microscopy and its bone forming characteristics were confirmed by performing alkaline phophatase staining on the cells and measuring the supernatant in which the cells grew for osteocalcin. This pure OS population of tumour cells (met-c738) from its corresponding parent tissue (met-738) had its RNA extracted after just two passages. The idea was to keep the tumour resembling the OS tissue as much as possible. If it underwent further passages it may change some of its characteristics by acquiring features consistent with an immortal cell line. Met-c738 was resuscitated from frozen successfully and underwent up to 5 passages without going into blast crisis. The RNA extracted for analysis was always taken at passage 2. Once this RNA was made into a cDNA probe it was used in the microarray analysis alongside the OS samples.

RNA extraction from OS tissue

My original research intention was to prospectively collect as many new OS samples as they became available from October 1999 from both the UCLH and RNOH sites. I wanted to demonstrate I could successfully extract RNA from the needle core biopsies as well as the more generous excision biopsy samples. OS is a very rare tumour with approximately 60 new cases per year presenting to the London Soft tissue and Bone sarcoma unit. I collected 4 new OS tissue samples between October 1999 and April 2000. I worked on these samples plus the archive tissue. In April 2000, an unexpected moratorium was placed on the collection of any new tissue for experimental use at the RNOH in response to the Alderhay Children's Hospital enquiry. Despite my project gaining prior ethical approval, it was a Trust policy to ensure procedures were in place to ensure all biological samples could be correctly accounted for. For the next nine months I worked through the previously stored samples and had no new fresh tissue to work with. It was not possible for any new OS cases to have surplus tissue stored for when the moratorium was lifted. In January 2001 my work was allowed to resume with the collection of new OS tissue. I had a total of 61 OS samples from which I attempted RNA extraction. 53 samples were collected in advance of starting my laboratory work. I therefore could not control the conditions of how these samples were collected to ensure RNA was not degraded. I successfully extracted RNA from 24 of these 53 archive samples, giving an extraction rate of 45%. My conclusion was that the samples were probably saved many hours after their removal from the patient without any attempt to preserve the RNA content such as the placing of samples on ice and rapidly transferring suitable tissue to sterile tubes for snap freezing. The London and Soft Tissue & Bone Tumour service encompasses both University College London Hospitals (UCLH) and The Royal National Orthopaedic Hospital (RNOH) some 12 miles apart. The histopathology

department which handles all the OS samples is based at RNOH. Samples coming direct from theatres or the radiology department at RNOH have just a short transfer time. Routine samples being transported from UCLH to RNOH can arrive the following working day. To ensure a more rapid delivery I formed a system with the sarcoma surgical team at UCLH and histopathologist at RNOH to ensure all new OS samples were correctly packaged and then stored on ice until their delivery at the pathology department at RNOH within one hour. All samples went through the routine checking and signing in by the pathology team at RNOH. Only if the consent had been signed and there was tissue surplus to diagnostic requirements was I given a separate sample from that patient to work with. That final decision was made by the Consultant Histopathologist. All eight samples personally collected by me and transported in this way yielded good quality RNA. An overall RNA extraction rate of 52% from all used samples was recorded but this belies the accurate picture when 100% extraction rate is possible from all collected samples under the correct conditions.

Feasibility of extracting RNA from needle core biopsy samples

Technically it was feasible to extract RNA from a thin core of OS from a needle biopsy as well as the dissected larger chunks of OS tissue taken at resection. More tissue meant several attempts were possible so there was more chance of a successful extraction. The thin core meant there was usually only one attempt to be successful. The core biopsies most commonly obtained pre-treatment often did not have enough surplus tissue for a spare core to be used for microarray analysis. I have demonstrated that it is technically feasible to obtain good quality RNA to generate a cDNA probe for microarray analysis from either a needle or core biopsy as long as the sample can be protected to prevent RNA degradation i.e. snap frozen within a few hours of removal

from the patient if immediate work to extract the RNA is not possible. There must be enough available surplus tissue after diagnostic procedures have been performed to allow release of the consented tissue for RNA analysis. This poses a huge potential problem for microarray work dependent on fresh tissue as patients may have to consent for extra samples purely for research purposes. Needle core biopsies particularly on young adults are not without discomfort. Several extra passes just to obtain tissue for research will undoubtedly cause extra distress. When microarray technology becomes a bedside tool to predict response to chemotherapy then the extra tissue required for this purpose will be directly of benefit to the patient so consent will be much more straightforward. The emerging technology which allows paraffin embedded samples to be used in place of fresh tissue in GEM work will be a large leap forward [270, 279-281].

Using extracted RNA to make cDNA probes

All extracted RNA that had a purity of 1.62 to 2.2 successfully generated cDNA probes. This amounted to 25 probes from the 30 RNA OS samples. To make replicates of each sample I needed 5μg of RNA. Replicates were possible from 12 of the 16 tissue samples as there was enough extracted RNA to perform this. 4 samples post-307, post-397, loc-181 and met-576 did not have enough extracted RNA to make replicate samples for the analysis. Three samples post-230, post-427 and post-265 made cDNA probes which were not of sufficient signal to be used in any hybridisations experiment to create a microarray. Samples post-124, post-850, post-610, post-605, post264 and post-290 which were used to make microarrays failed to meet the pre-determined statistical minimum data set so were excluded from further analysis.

A replicate is defined as a cDNA probe made from the same RNA sample at the same time. A duplicate is a cDNA probe made from a different RNA sample of the same tissue. To illustrate: if I had enough surplus OS tissue from any of my samples ideally I would have extracted RNA at different times to optimise the accuracy of the data generated. This of course was not possible. In the majority of the samples I was able to make replicates. This meant I could average the replicate results from each of the cDNA probes generated to increase the accuracy of the data before assessing differential gene expression between samples. Only replicates which had a high correlation co-efficient could be used for this purpose.

As this work did not involve any time course experimentation, the use of replicates alone and not duplicates can be justified.

OS Gene expression microarray data

In this pilot study I used nylon filter microarrays on which 5,184 known human genes are represented by inert cDNA clones. I obtained data from filters provided by Research Genetics® for 16 OS tumour samples and 2 commercially established OS cell.

The Human Genome project has so far predicted approximately 30,000 genes [282]. I have therefore looked at almost 15% of the total identified human genes to date in our microarray analysis osteosarcoma tissue. In addition although Osteosarcoma is one of the most common solid tumours to affect teenagers it still remains a rare clinical entity.

The inclusion of two well characterised OS cell lines represents an internal cross-validation control. I have accurately replicated the p53 and RB status of these two cell lines with the vast amount of published data on their characteristics [250, 254, 283].

The samples divide into three main clusters A, B and C. The expression profile of Saos 2 lies outside all other samples but is linked to all. All samples bar one have been collected from young patients with the exception of sample pre-124 which was taken from a 71 year old female. The clusters did not divide neatly into primary and secondary sites but appear to be separated on the basis of whether they had received chemotherapy immediately prior to resection or not. Cluster B which contained 10 tissue samples, 9 of which had all received immediate pre-operative chemotherapy. The majority of the samples are primaries but two metastases fall into this cluster. The expression profile for the cell line MG63 clusters within B. Looking at reasons which might account for this, the groups of genes which are differentially expressed between these two clusters provide some clues. Genes which are significantly up regulated in cluster B compared with cluster A include enzymes responsible for the detoxification and protection of cells.

Samples which bear the more similar profiles will cluster closer together. The two different probes made from the tumour tissue met-738 clustered together despite the fact one was generated from cultured cells from this tissue and the other obtained by extracting RNA from the whole tissue. A similar observation was made by Perou et al, in that it was noted independent samples taken from the same tumour were in most cases recognisably more similar to each other than either was to any of the other samples [225]. It was also noted in this paper that a metastasis and primary tumour were as similar in their overall pattern of gene expression as were repeated samplings

of the same primary tumour. From this it was inferred that perhaps the molecular make up of the primary tumour was retained in its metastasis.

The very red area which reflects the highly up regulated genes in all tumour samples contained many bone markers (Table 3.3). In particular, Collagen-1, alpha-2 and Matrix Gla are the major constituents of bone matrix and reinforces the high osteoid content of all samples. The expression of Ezrin was just outside the 99th percentile and is of particular interest due to its previously reported association of OS metastasis in an OS cell line [252]. Finally the group of genes universally down-regulated compared to our reference sample include Vimentin, endothelin1 and keratin 18. This most likely reflects the make up of the reference probe compared to the tumour samples. By creating a ratio of expression between tumour sample and a reference made up endothelial cells, human osteoblasts and peripheral blood mononuclear cells we tried to effectively screen out any "contaminating" stromal/supporting cells. Although p53 is amongst this group its expression is only just 1.88 fold less than the tumour samples so I am not sure we can infer any meaningful conclusion from this data set.

When I first started this project in October 1999 publications involving gene expression microarray technology were few. The available affordable microarray systems with which I could work were restricted to the use of the paper style Research Genetic® microarray system which I used. Since this work was conceived and completed, a number of publications have tried to demonstrate an expression signature for OS. Up until recently GEM work in OS has focussed on OS cell lines but two groups have now published their work using biopsy samples. Ochi et al have

performed an expression profile on 19 biopsy samples and demonstrated two major clusters. The major cluster in their work comprised individuals who were affected by conventional central OS in their long bones all in the second decade of life. They also determined a set of 60 genes whose expression was most likely to be associated with response to chemotherapy [284]. Mintz et al used Affymetrix oligonucleotides arrays on 30 OS diagnostic samples. They discovered a class of 104 genes differentially expressed between Huvos I/II and Huvos III/IV patients. Markers of Huvos I/II were predominantly gene products in the extracellular matrix microenvironment remodelling and osteoclast differentiation groups. This finding led to their conclusion that OS tumour driven changes in the bone microenvironment contribute to the chemoresistant phenotype [285]. If these findings are validated by future studies our drive to exclude "contaminating" cells in the tissue may well have led to the loss of important data from cells contained in the tumour microenvironment.

In principle there are two ways of using data from tumour samples after microarray analyses: firstly, one can systematically search for single genes that may be related to the prognosis, or that may be useful as therapeutic targets. The second is to use the whole set of expressed genes to classify tumours. For example, this type of approach has been used to predict outcome in women with breast cancer to determine "good" and "poor" prognosis patients [286-300]. When compared to the classic prognostic indicators such as grade, stage and nodal status in a multivariate analysis the gene expression fingerprint performed better. Increasingly, gene expression profiling will be used to determine prognosis and the most appropriate treatment (214-227). Gene expression microarrays are powerful, but variability throughout the measurement process could potentially obscure biologically meaningful data. Technological errors may be small, but can occur at any time from the preparation of mRNA from

biological samples through to the amplification, purification and concentration of cDNA clones. During hybridisation variability can arise from the laboratory, during scanning natural fluorescence and non-specific binding can increase background noise, making interpretation of what is real difficult. A major obstacle for the widespread introduction of these new technologies is the current requirement for fresh tissue. Although the use of GEM's from archived paraffin embedded tissues are now being optimised [270], this is not yet standardised for widespread use. The high cost, and complex experimental artefacts have also emphasised the need for statistical monitoring through all stages of the experiment.

cDNA microarray technology, with the simultaneous analysis of thousands of genes in just one experiment, has already provided us with new information on major common tumour types [231, 235, 296, 301-307]. The detailed molecular expression profiles of individual cancers (signatures) could provide new classification algorithms, as well as novel tumour markers which may allow better prediction of clinical behaviour and new targets for therapeutic intervention [307].

Summary

The aim of this work was to determine whether needle and open biopsies from osteosarcoma (OS) provide sufficient quality of mRNA for cDNA array analyses which might then provide insights into the expression profile of OS. I have demonstrated if specimens are correctly collected then it is feasible to obtain enough good quality RNA, without amplification of RNA, to make cDNA probes to generate data suitable for interpretation in a gene expression microarray dataset. I have also shown that OS tissue including small needle core bone biopsies, are amenable to

cDNA array analysis. It was unfortunate that the publicity from Alderhey Children's Hospital led to a moratorium on the collection and storage of new samples at RNOH for nine months. My total research period for this work from start to finish was two years. My original aim in this pilot work was to collect both pre and post treatment samples on all consenting patients to see if I could predict prognostic information from the gene expression data. Although I was not able to do this I have been able to obtain gene expression data on 16 patient samples plus the two OS cell lines. Compared to the most recent published work on OS this is a reasonable sample size. The major drawback which prevents further conclusions being drawn from my profile data is that the samples are predominantly from the poorest prognostic group. Apart from the three pre-treatment samples the remainder of the analysed samples are from patients whose tumours have sustained a poor histological response to cytotoxic chemotherapy or those who have already relapsed. Some novel genes have been identified in this work in addition to the identification of several well known "bone" genes. The up-regulation of genes associated with detoxification of the cells or enzymes associated with drug resistance would be consistent with this cohort of patients, however without a good prognostic group to compare I accept I cannot draw any further conclusions but instead accept the foundations are laid for future work to build on. Performing RT-PCR and immunohistochemistry on some of the corresponding archived tissue samples was undertaken to validate the array data. The PCR products confirmed that the expression level obtained was from mRNA from the OS sample and not contaminating DNA. The protein expression level detected by immunohistochemistry on the corresponding tissue samples confirmed the increased level of mRNA led to the formation of the corresponding protein. The reason I chose ezrin for the validatory work was based on Khanna et al proposal that ezrin is implicated in the development of metastases in OS [257]. Their work was performed on murine cell lines and OS in

dogs but felt the results could be elucidated to human OS. My array data shows the expression level of ezrin for all samples is just outside the 99th percentile. Given my samples are predominantly from patients with the poorest outlook this could be an interesting finding. This along with the difference in cellular distribution of ezrin between the primary and metastatic samples is certainly worthy of further study in OS.

This work was started at a time when microarray technology was in its infancy. I used the most affordable system available. This system has now been superseded by the oligonucletide arrays such as Affymetrix™. Although both systems exploit hybridisation they differ in how the DNA sequences are laid out, the length of these sequences and the sheer number of genes available per chip. The Affymetrix GeneChip™ type arrays contain up to 30,000 genes.

Picking an appropriate reference sample against which the tissue of interest can be measured is an on-going problem. I was originally going to compare OS with human osteoblasts but after a personal communication with Dr Charles Perou, Stanford University School of Medicine, Stanford, California, I compared the OS samples to a reference pool made from a mixture of endothelial cells, osteoblasts and peripheral mononuclear cells. The rationale behind this was that OS is not just made up from pure malignant cells derived from osteoblasts but instead recruits other cell types from the microenvironment. These are referred to as host cells. By creating a reference sample enriched for such host cells it was anticipated that such "contaminating cells" could be excluded from analysis. This would allow only the genes expressed by the OS to be accurately measured. There is no doubt looking at the set of down-regulated genes in this work that the reference sample excluded whole sets of such genes but my concern in pursuing this approach is that I may have inadvertently lost important data

as described by the Mintz publication [285]. Similarly the process of enriching for OS cells by the use of a primary cell culture would lose potentially important data as host cells are not able to sustain life in cell culture conditions.

Finally, the method of hierarchical clustering used in this project is just one of the ways to represent and make sense of the gene expression data generated. The growing complexity of technologies now available for high-throughput gene expression array data means many more specific statistical modelling tools have been developed. Choosing the most appropriate statistical approach is paramount in ensuring the data generated is correctly interpreted. Thanks to Dr Stephen Henderson, Bioinformaticist at UCL I have been able to generate some meaningful results from this dataset and establish some genes of interest for future work in this group.

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Appendix 1 - Patient Information Sheet

PATIENT INFORMATION SHEET

The use of tumour and blood samples for scientific analysis of osteosarcoma

We would like you and your child to help us study your cancer by allowing us to use some of your tumour and blood for scientific analysis.

You/your child is about to have a procedure called a biopsy for taking out a sample of your suspected cancer to confirm that it is an osteosarcoma.. Although the treatment of osteosarcoma has improved there is still a lot to learn about this rare bone cancer. We are currently using a new technology which analyses all the genes that are contained in your tumour. This means we can learn the unique information contained in your/your child's tumour which instructs it how to behave.

We would also like to take a sample of your tumour if possible after you have your chemotherapy treatment. Using the same technology we can learn if the chemotherapy has changed any of its information we call its genetic expression.

We will only take some of your tumour if there is spare tissue left after your diagnosis is made. Your treatment takes priority and in no way will this be affected by the study.

The sample of you/your child's tumour will be carefully stored in a special freezer. A unique number will be given to it identify it in the storage freezer.

We hope in time if we can analyse enough samples of this rare bone tumour we can help individualise your cancer treatment so maximising your chance of a cure.

If you/ your child change their mind and wish the tissue not to be used for this research project, then as soon as you have told your doctor any stored tissue will be destroyed.

If you would like any further information please contact:

Dr Jeremy Whelan
Consultant in Medical Oncology
University College London Hospitals NHS Trust
Meyerstein Institute of Oncology
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Appendix 2 – BJC Osteosarcoma Paper

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